

BEST AVAILABLE COPY



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Moritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

B 7 - 2 : CTL A4/CD 28 COUNTER RECEPTOR

Government Funding

Work described herein was supported under CA-40216-08 awarded by the National
5 Institutes of Health. The U.S. government therefore may have certain rights in this invention.

Background of the Invention

To induce antigen-specific T cell activation and clonal expansion, two signals provided by antigen-presenting cells (APCs) must be delivered to the surface of resting T 10 lymphocytes (Jenkins, M. and Schwartz, R. (1987) *J. Exp. Med.* **165**, 302-319; Mueller, D.L., et al. (1990) *J. Immunol.* **144**, 3701-3709; Williams, I.R. and Unanue, E.R. (1990) *J. Immunol.* **145**, 85-93). The first signal, which confers specificity to the immune response, is mediated via the T cell receptor (TCR) following recognition of foreign antigenic peptide presented in the context of the major histocompatibility complex (MHC). The second signal, 15 termed costimulation, induces T cells to proliferate and become functional (Schwartz, R.H. (1990) *Science* **248**, 1349-1356). Costimulation is neither antigen-specific, nor MHC restricted and is thought to be provided by one or more distinct cell surface molecules expressed by APCs (Jenkins, M.K., et al. (1988) *J. Immunol.* **140**, 3324-3330; Linsley, P.S., et al. (1991) *J. Exp. Med.* **173**, 721-730; Gimmi, C.D., et al., (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6575-6579; Young, J.W., et al. (1992) *J. Clin. Invest.* **90**, 229-237; Koulova, L., et al. (1991) *J. Exp. Med.* **173**, 759-762; Reiser, H., et al. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 271-275; van-Seventer, G.A., et al. (1990) *J. Immunol.* **144**, 4579-4586; LaSalle, J.M., et al., (1991) *J. Immunol.* **147**, 774-80; Dustin, M.I., et al., (1989) *J. Exp. Med.* **169**, 503; Armitage, R.J., et al. (1992) *Nature* **357**, 80-82; Liu, Y., et al. (1992) *J. Exp. Med.* **175**, 437-445).

20 Considerable evidence suggests that the B7 protein, expressed on APCs, is one such critical costimulatory molecule (Linsley, P.S., et al., (1991) *J. Exp. Med.* **173**, 721-730; Gimmi, C.D., et al., (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6575-6579; Koulova, L., et al., (1991) *J. Exp. Med.* **173**, 759-762; Reiser, H., et al. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 271-275; Linsley, P.S. et al. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5031-5035; Freeman, G.J. et al. (1991) *J. Exp. Med.* **174**, 625-631.). B7 is the counter-receptor for two ligands 25 expressed on T lymphocytes. The first ligand, termed CD28, is constitutively expressed on resting T cells and increases after activation. After signaling through the T cell receptor, ligation of CD28 induces T cells to proliferate and secrete IL-2 (Linsley, P.S., et al. (1991) *J. Exp. Med.* **173**, 721-730; Gimmi, C.D., et al. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6575-6579; Thompson, C.B., et al. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1333-1337; June, C.H., et al. (1990) *Immunol. Today* **11**, 211-6; Harding, F.A., et al. (1992) *Nature* **356**, 607-609.). The second ligand, termed CTLA4 is homologous to CD28 but is not expressed on resting T 30 cells and appears following T cell activation (Brunet, J.F., et al., (1987) *Nature* **328**, 267-270). DNA sequences encoding the human and murine CTLA4 protein are described in

-2-

Dariavich, et al. (1988) *Eur. J. Immunol.* **18**(12), 1901-1905; Brunet, J.F., et al. (1987) *supra*; Brunet, J.F. et al. (1988) *Immunol. Rev.* **103**:21-36; and Freeman, G.J., et al. (1992) *J. Immunol.* **149**, 3795-3801. Although B7 has a higher affinity for CTLA4 than for CD28 (Linsley, P.S., et al., (1991) *J. Exp. Med.* **174**, 561-569), the function of CTLA4 is still 5 unknown.

The importance of the B7:CD28/CTLA4 costimulatory pathway has been demonstrated *in vitro* and in several *in vivo* model systems. Blockade of this costimulatory pathway results in the development of antigen specific tolerance in murine and humans systems (Harding, F.A., et al. (1992) *Nature* **356**, 607-609; Lenschow, D.J., et al. (1992) *Science* **257**, 789-792; Turka, L.A., et al. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11102-11105; Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci USA* **90**, 6586-6590; Boussiotis, V., et al. (1993) *J. Exp. Med.* **178**, 1753-1763). Conversely, expression of B7 by B7 negative murine tumor cells induces T-cell mediated specific immunity accompanied by tumor rejection and long lasting protection to tumor challenge (Chen, L., et al. (1992) *Cell* **71**, 1093-1102; Townsend, S.E. and Allison, J.P. (1993) *Science* **259**, 368-370; Baskar, S., et al. (1993) *Proc. Natl. Acad. Sci.* **90**, 5687-5690.). Therefore, manipulation of the B7:CD28/CTLA4 pathway offers great potential to stimulate or suppress immune responses in humans.

20 **Summary of the Invention**

This invention pertains to isolated nucleic acids encoding novel molecules which costimulate T cell activation. Preferred costimulatory molecules include antigens on the surface of B lymphocytes, professional antigen presenting cells (e.g., monocytes, dendritic cells, Langerhan cells) and other cells (e.g., keratinocytes, endothelial cells, astrocytes, 25 fibroblasts, oligodendrocytes) which present antigen to immune cells, and which bind either CTLA4, CD28, both CTLA4 and CD28 or other known or as yet undefined receptors on immune cells. Such costimulatory molecules are referred to herein as CTLA4/CD28 binding counter-receptors or B lymphocyte antigens, and are capable of providing costimulation to activated T cells to thereby induce T cell proliferation and/or cytokine secretion. Preferred B 30 lymphocyte antigens include B7-2 and B7-3 and soluble fragments or derivatives thereof which bind CTLA4 and/or CD28 and have the ability to inhibit or induce costimulation of immune cells. In one embodiment, an isolated nucleic acid which encodes a peptide having the activity of the human B7-2 B lymphocyte antigen is provided. Preferably, the nucleic acid is a cDNA molecule having a nucleotide sequence encoding human B7-2, as shown in 35 Figure 8 (SEQ ID NO:1). In another embodiment, the nucleic acid is a cDNA molecule having a nucleotide sequence encoding murine B7-2, as shown in Figure 14 (SEQ ID NO:22).

-3-

The invention also features nucleic acids which encode a peptide having B7-2 activity and at least about 50%, more preferably at least about 60% and most preferably at least about 70% homologous with an amino acid sequence shown in Figure 8 (SEQ ID NO:2) or an amino acid sequence shown in Figure 14 (SEQ ID NO:23). Nucleic acids which encode 5 peptides having B7-2 activity and at least about 80%, more preferably at least about 90%, more preferably at least about 95% and most preferably at least about 98% or at least about 99% homologous with an amino acid sequence shown in Figure 8 (SEQ ID NO:2) or an amino acid sequence shown in Figure 14 (SEQ ID NO:23) are also within the scope of the invention. In another embodiment, the peptide having B7-2 activity is encoded by a nucleic 10 acid which hybridizes under high or low stringency conditions to a nucleic acid which encodes a peptide having an amino acid sequence of Figure 8 (SEQ ID NO:2) or a peptide having an amino acid sequence shown in Figure 14 (SEQ ID NO:23).

The invention further pertains to an isolated nucleic acid comprising a nucleotide sequence encoding a peptide having B7-2 activity and having a length of at least 20 amino 15 acid residues. Peptides having B7-2 activity and consisting of at least 40 amino acid residues in length, at least 60 amino acid residues in length, at least 80 amino acid residues in length, at least 100 amino acid residues in length or at least 200 or more amino acid residues in length are also within the scope of this invention. Particularly preferred nucleic acids encode a peptide having B7-2 activity, a length of at least 20 amino acid residues or more and at least 20 50% or greater homology (preferably at least 70%) with a sequence shown in Figure 8 (SEQ ID NO:2).

In one preferred embodiment, the invention features an isolated DNA encoding a peptide having B7-2 activity and an amino acid sequence represented by a formula:

25 X_n-Y-Z_m

In the formula, Y consists essentially of amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2). X_n and Z_m are additional amino acid residue(s) linked to Y by an amide bond. X_n and Z_m are amino acid residues selected from amino acid residues 30 contiguous to Y in the amino acid sequence shown in Figure 8 (SEQ ID NO:2). X_n is amino acid residue(s) selected from amino acids contiguous to the amino terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2), i.e., selected from amino acid residue 23 to 1. Z_m is amino acid residue(s) selected from amino acids contiguous to the carboxy terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2), i.e., selected from amino acid residue 35 246 to 329. According to the formula, n is a number from 0 to 23 (n=0-23) and m is a number from 0 to 84 (m=0-84). A particularly preferred DNA encodes a peptide having an

-4-

amino acid sequence represented by the formula X_n-Y-Z_m , where Y is amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2) and n=0 and m=0.

The invention also features an isolated DNA encoding a B7-2 fusion protein which includes a nucleotide sequence encoding a first peptide having B7-2 activity and a nucleotide 5 sequence encoding a second peptide corresponding to a moiety that alters the solubility, binding affinity, stability or valency of the first peptide. Preferably, the first peptide having B7-2 activity includes an extracellular domain portion of the B7-2 protein (e.g., about amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2)) and the second peptide is an immunoglobulin constant region, for example, a human Cy1 or Cy4 domain, 10 including the hinge, CH2 and CH3 region, to produce a B7-2 immunoglobulin fusion protein (B7-2Ig)(see Capon et al. (1989) *Nature* 337, 525-531 and Capon U.S. 5,116,964).

The nucleic acids obtained in accordance with the present invention can be inserted into various expression vectors, which in turn direct the synthesis of the corresponding protein or peptides in a variety of hosts, particularly eucaryotic cells, such as mammalian and 15 insect cell culture, and prokaryotic cells such as *E. coli*. Expression vectors within the scope of the invention comprise a nucleic acid encoding at least one peptide having the activity of a novel B lymphocyte antigen as described herein, and a promoter operably linked to the nucleic acid sequence. In one embodiment, the expression vector contains a DNA encoding a peptide having the activity of the B7-2 antigen and a DNA encoding a peptide having the 20 activity of another B lymphocyte antigen, such as the previously characterized B7 activation antigen, referred to herein as B7-1. Such expression vectors can be used to transfect host cells to thereby produce proteins and peptides, including fusion proteins, encoded by nucleic acids as described herein.

Nucleic acid probes useful for assaying a biological sample for the presence of B cells 25 expressing the B lymphocyte antigens B7-2 and B7-3 are also within the scope of the invention.

The invention further pertains to isolated peptides having the activity of a novel B lymphocyte antigen, including the B7-2 and B7-3 protein antigens. A preferred peptide having B7-2 activity is produced by recombinant expression and comprises an amino acid 30 sequence shown in Figure 8 (SEQ ID NO: 2). Another preferred peptide having B7-2 activity comprises an amino acid sequence shown in Figure 14 (SEQ ID NO:23). A particularly preferred peptide having the activity of the B7-2 antigen includes at least a portion of the mature form of the protein, such as an extracellular domain portion (e.g., about amino acid residues 24-245 of SEQ ID NO:2) which can be used to enhance or suppress T-cell mediated 35 immune responses in a subject. Other preferred peptides having B7-2 activity include peptides having an amino acid sequence represented by a formula:

-5-

X_n-Y-Z_m

In the formula, Y is amino acid residues selected from the group consisting of: amino acid residues 55-68 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 81-89 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 128-142 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 160-169 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 188-200 of the sequence shown in Figure 8 (SEQ ID NO:2); and amino acid residues 269-282 of the sequence shown in Figure 8 (SEQ ID NO:2). In the formula X_n and Z_m are additional amino acid residue(s) linked to Y by an amide bond and are selected from amino acid residues contiguous to Y in the amino acid sequence shown in Figure 8 (SEQ ID NO:2). X_n is amino acid residue(s) selected from amino acids contiguous to the amino terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2). Z_m is amino acid residue(s) selected from amino acids contiguous to the carboxy terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2). According to the formula, 10 n is a number from 0 to 30 (n=0-30) and m is a number from 0 to 30 (m=0-30).

Fusion proteins or hybrid fusion proteins including a peptide having the activity of a novel B lymphocyte antigen (e.g., B7-2, B7-3) are also featured. For example, a fusion protein comprising a first peptide which includes an extracellular domain portion of a novel B lymphocyte antigen fused to second peptide, such as an immunoglobulin constant region, that 20 alters the solubility, binding affinity, stability and/or valency of the first peptide are provided. In one embodiment, a fusion protein is produced comprising a first peptide which includes amino acid residues of an extracellular domain portion of the B7-2 protein joined to a second peptide which includes amino acid residues of a sequence corresponding to the hinge, CH2 and CH3 regions of Cy1 or Cy4 to form a B7-2Ig fusion protein. In another embodiment, a 25 hybrid fusion protein is produced comprising a first peptide which includes an extracellular domain portion of the B7-1 antigen and an extracellular domain portion of the B7-2 antigen and a second peptide which includes amino acid residues corresponding to the hinge, CH2 and CH3 of Cy1 (see e.g., Linsley et al. (1991) *J. Exp. Med.* 173:721-730; Capon et al. (1989) *Nature* 337, 525-531; and Capon U.S. 5,116,964).

30 Isolated peptides and fusion proteins of the invention can be administered to a subject to either upregulate or inhibit the expression of one or more B lymphocyte antigens or block the ligation of one or more B lymphocyte antigens to their natural ligand on immune cells, such as T cells, to thereby provide enhancement or suppression of cell-mediated immune responses *in vivo*.

35 Another embodiment of the invention provides antibodies, preferably monoclonal antibodies, specifically reactive with a peptide of a novel B lymphocyte antigen or fusion protein as described herein. Preferred antibodies are anti-human B7-2 monoclonal antibodies

-6-

produced by hybridoma cells HF2.3D1, HA5.2B7 and HA3.1F9. These hybridoma cells have been deposited with the American Type Culture Collection at ATCC Accession No.____ (HF2.3D1), ATCC Accession No.____ (HA5.2B7), and ATCC Accession No.____ (HA3.1F9).

5 A still further aspect of the invention involves the use of the nucleic acids of the invention, especially the cDNAs, to enhance the immunogenicity of a mammalian cell. In preferred embodiments, the mammalian cell is a tumor cell, such as a sarcoma, a lymphoma, a melanoma, a neuroblastoma, a leukemia or a carcinoma, or an antigen presenting cell, such as a macrophage, which is transfected to allow expression of a peptide having the activity of a
10 novel B lymphocyte antigen of the invention on the surface of the cell. Macrophages that express a peptide having the activity of a B lymphocyte antigen, such as the B7-2 antigen, can be used as antigen presenting cells, which, when pulsed with an appropriate pathogen-related antigen or tumor antigen, enhance T cell activation and immune stimulation.

Mammalian cells can be transfected with a suitable expression vector containing a
15 nucleic acid encoding a peptide having the activity of a novel B lymphocyte antigen, such as the B7-2 antigen, *ex vivo* and then introduced into the host mammal, or alternatively, cells can be transfected with the gene *in vivo* via gene therapy techniques. For example, the nucleic acid encoding a peptide having B7-2 activity can be transfected alone, or in combination with nucleic acids encoding other costimulatory molecules. In enhancing the
20 immunogenicity of tumors which do not express Class I or Class II MHC molecules, it may be beneficial to additionally transfect appropriate class I or II genes into the mammalian cells to be transfected with a nucleic acid encoding a peptide having the activity of a B lymphocyte antigen, as described herein.

The invention also provides methods for inducing both general immunosuppression
25 and antigen-specific tolerance in a subject by, for example, blocking the functional interaction of the novel B lymphocyte antigens of the invention, e.g., B7-2 and B7-3, to their natural ligand(s) on T cells or other immune system cells, to thereby block co-stimulation through the receptor-ligand pair. In one embodiment, inhibitory molecules that can be used to block the interaction of the natural human B7-2 antigen to its natural ligands (e.g., CTLA4
30 and CD28) include a soluble peptide having B7-2 binding activity but lacking the ability to costimulate immune cells, antibodies that block the binding of B7-2 to its ligands and fail to deliver a co-stimulatory signal (so called "blocking antibodies", such as blocking anti-B7-2 antibodies), B7-2-Ig fusion proteins, which can be produced in accordance with the teachings of the present invention, as well as soluble forms of B7-2 receptors, such as CTLA4Ig or
35 CD28Ig. Such blocking agents can be used alone or in combination with agents which block interaction of other costimulatory molecules with their natural ligands (e.g., anti-B7 antibody). Inhibition of T cell responses and induction of T cell tolerance according to the

-7-

methods described herein may be useful prophylactically, in preventing transplantation rejection (solid organ, skin and bone marrow) and graft versus host disease, especially in allogeneic bone marrow transplantation. The methods of the invention may also be useful therapeutically, in the treatment of autoimmune diseases, allergy and allergic reactions, 5 transplantation rejection, and established graft versus host disease in a subject.

Another aspect of the invention features methods for upregulating immune responses by delivery of a costimulatory signal to T cells through use of a stimulatory form of B7-2 antigen, which include soluble, multivalent forms of B7-2 protein, such as a peptide having B7-2 activity and B7-2 fusion proteins. Delivery of a stimulatory form of B7-2 in 10 conjunction with antigen may be useful prophylactically to enhance the efficacy of vaccination against a variety of pathogens and may also be useful therapeutically to upregulate an immune response against a particular pathogen during an infection or against a tumor in a tumor-bearing host.

The invention also features methods of identifying molecules which can inhibit either 15 the interaction of B lymphocyte antigens, e.g., B7-2, B7-3, with their receptors or interfere with intracellular signalling through their receptors. Methods for identifying molecules which can modulate the expression of B lymphocyte antigens on cells are also provided. In addition, methods for identifying cytokines produced in response to costimulation of T cells by novel B lymphocyte antigens are within the scope of the invention.

20

Brief Description of the Drawings

Figure 1A-B are graphic representations of the responses of CD28⁺ T cells, as assessed by ³H-thymidine incorporation or IL-2 secretion, to costimulation provided by either B7 (B7-1) transfected CHO cells (panel a) or syngeneic activated B lymphocytes 25 (panel b) cultured in media, anti-CD3 alone, or anti-CD3 in the presence of the following monoclonal antibodies or recombinant proteins: α B7 (133, anti-B7-1); CTLA4Ig; Fab α CD28; control Ig fusion protein (isotype control for CTLA4Ig); or α B5 (anti-B5, the isotype control for anti-B7-1).

Figure 2A-C are graphs of log fluorescence intensity of cell surface expression of 30 B7-1 on splenic B cells activated with surface immunoglobulin (slg) crosslinking. The total (panel a), B7-1 positive (B7-1⁺, panel b) and B7-1 negative (B7-1⁻, panel c) activated B cells were stained with anti-B7-1 monoclonal antibody (133) and fluorescein isothiocyanate (FITC) labeled goat anti-mouse immunoglobulin and analyzed by flow cytometry.

Figure 3A-B are graphic representations of the responses of CD28⁺ T cells, as 35 assessed by ³H-thymidine incorporation and IL-2 secretion, to costimulation provided by B7-1⁺ (panel a) or B7-1⁻ (panel b) activated syngeneic B lymphocytes cultured in media, anti-CD3 alone, or anti-CD3 in the presence of the following monoclonal antibodies or

-8-

recombinant proteins: α BB-1 (133, anti-B7-1 and anti-B7-3); α B7 (anti-B7-1); CTLA4Ig; Fab α CD28; control Ig fusion protein or α B5 (anti-B5).

Figure 4 is a graphic representation of the cell surface expression of B7-1, B7-3 and total CTLA4 counter-receptors on fractionated B7-1⁺ and B7-1⁻ activated B lymphocytes.

5 Figure 5 is a graphic representation of temporal surface expression of B7-1 (CTLA4Ig and mAbs BB-1 and 133), B7-3 (CTLA4Ig and mAb BB1) and B7-2 (CTLA4Ig) counter-receptors on splenic B cells activated by sIg crosslinking.

10 Figure 6 is a graphic representation of temporal surface expression of B7-1 (CTLA4Ig and mAbs BB-1 and 133), B7-3 (CTLA4Ig and mAb BB1) and B7-2 (CTLA4Ig) counter-receptors on splenic B cells activated by MHC class II crosslinking.

15 Figure 7A-B are graphic representations of the response of CD28⁺ T cells, as assessed by ³H-thymidine incorporation and IL-2 secretion, to costimulation provided by syngeneic B lymphocytes activated by sIg crosslinking for 24 hours (panel a) or 48 hours (panel b) and cultured in media, anti-CD3 alone, or anti-CD3 in the presence of the following monoclonal antibodies or recombinant protein: α B7(133, anti-B7-1); α BB1 (anti-B7-1, anti-B7-3) CTLA4Ig; Fab α CD28; and α B5(anti-B5).

Figure 8 is the nucleotide and deduced amino acid sequence of the human B lymphocyte antigen B7-2 (hB7-2-clone29).

20 Figure 9 is a graphic representation of COS cells transfected with control plasmid (pCDNA1), plasmid expressing B7-1 (B7-1), or plasmid expressing B7-2 (B7-2) stained with either control mAb (IgM), anti-B7-1 (mAbs 133 and BB-1), recombinant protein CTLA4Ig, or isotype matched control Ig protein followed by the appropriate second FITC labelled immunoglobulin and analyzed by flow cytometry.

25 Figure 10A-B show RNA blot analyses of B7-2 expression in unstimulated and anti-Ig activated human spenic B cells and cell lines (panel a) and human myelomas (panel b).

Figure 11 is a graphic representation of the proliferation of CD28⁺ T cells, as assessed by ³H-thymidine incorporation or IL-2 secretion, to submitogenic stimulation with phorbol myristic acid (PMA) and COS cells transfected with vector alone or vectors directing the expression of either B7-1 or B7-2.

30 Figure 12 is a graphic representation of the inhibition by mAbs and recombinant proteins of the proliferation of CD28⁺ T cells, as assessed by ³H-thymidine incorporation and IL-2 secretion, to stimulation by PMA and COS cells transfected with vector alone (vector), or with a vector expressing B7-1 (B7-1) or B7-2 (B7-2). Inhibition studies were performed with the addition of either no antibody (no mAb), anti-B7 mAb 133 (133), anti-B7 mAb BB-1 (BB1), anti-B5 mAb (B5), Fab fragment of anti-CD28 (CD28 Fab), CTLA4Ig (CTLA4Ig), or Ig control protein (control Ig) to the PMA stimulated COS cell admixed CD28⁺ T cells.

-9-

Figure 13 shows the sequence homology between the human B7-2 protein (h B7-2) deduced amino acid sequence (SEQ ID NO: 2) and the amino acid sequence of both the human B7-1 protein (h B7-1) (SEQ ID NO: 28 and 29) and the murine B7-1 protein (m B7) (SEQ ID NO: 30 and 31).

5 *Figure 14* is the nucleotide and deduced amino acid sequence of the murine B7-2 antigen (mB7-2) (SEQ ID NO: 22 and 23).

10 *Figure 15* is a graphic representation of the competitive inhibition of binding of biotinylated-CTLA4Ig to immobilized B7-2 Ig by B7 family-Ig fusion proteins. The Ig fusion proteins examined as competitors were: full-length B7-2 (hB7.2), full-length B7-1 (hB7.1), the variable region-like domain of B7-2 (hB7.2V) or the constant region-like domain of B7-2 (hB7.2C).

15 *Figure 16A-B* are graphic representations of the competitive inhibition of binding of biotinylated-B7-1-Ig (panel A) or B7-2-Ig (panel B) to immobilized CTLA4-Ig by increasing concentrations of unlabelled B7-1-Ig (panel A) or B7-2-Ig (panel B). The experimentally determined IC₅₀ values are indicated in the upper right corner of the panels.

20 *Figure 17* depicts flow cytometric profiles of cells stained with an anti-hB7-2 monoclonal antibody, HA3.1F9. Cells stained with the antibody were CHO cells transfected to express human B7-2 (CHO-hB7.2), NIH 3T3 cells transfected to express human B7-2 (3T3-hB7.2) and control transfected NIH 3T3 cells (3T3-neo). The anti-hB7.2 antibody B70 was used as a positive control.

25 *Figure 18* depicts flow cytometric profiles of cells stained with an anti-hB7-2 monoclonal antibody, HA5.2B7. Cells stained with the antibody were CHO cells transfected to express human B7-2 (CHO-hB7.2), NIH 3T3 cells transfected to express human B7-2 (3T3-hB7.2) and control transfected NIH 3T3 cells (3T3-neo). The anti-hB7.2 antibody B70 was used as a positive control.

30 *Figure 19* depicts flow cytometric profiles of cells stained with an anti-hB7-2 monoclonal antibody, HF2.3D1. Cells stained with the antibody were CHO cells transfected to express human B7-2 (CHO-hB7.2), NIH 3T3 cells transfected to express human B7-2 (3T3-hB7.2) and control transfected NIH 3T3 cells (3T3-neo). The anti-hB7.2 antibody B70 was used as a positive control.

35 *Figure 20* is a graphic representation of tumor cell growth (as measured by tumor size) in mice following transplantation of J558 plasmacytoma cells or J558 plasmacytoma cells transfected to express B7-1 (J558-B7.1) or B7-2 (J558-B7.2).

35 **Detailed Description of the Invention**

In addition to the previously characterized B lymphocyte activation antigen B7 (referred to herein as B7-1), human B lymphocytes express other novel molecules which

-10-

costimulate T cell activation. These costimulatory molecules include antigens on the surface of B lymphocytes, professional antigen presenting cells (e.g., monocytes, dendritic cells, Langerhan cells) and other cells (e.g., keratinocytes, endothelial cells, astrocytes, fibroblasts, oligodendrocytes) which present antigen to immune cells, and which bind either CTLA4, 5 CD28, both CTLA4 and CD28 or other known or as yet undefined receptors on immune cells. Costimulatory molecules within the scope of the invention are referred to herein as CTLA4/CD28 ligands (counter-receptors) or B lymphocyte antigens. Novel B lymphocyte antigens which provide cotimulation to activated T cells to thereby induce T cell proliferation and/or cytokine secretion include the B7-2 (human and murine) and the B7-3 antigens 10 described and characterized herein.

The B lymphocyte antigen B7-2 is expressed by human B cells at about 24 hours following stimulation with either anti-immunoglobulin or anti-MHC class II monoclonal antibody. The B7-2 antigen induces detectable IL-2 secretion and T cell proliferation. At about 48 to 72 hours post activation, human B cells express both B7-1 and a third CTLA4 15 counter-receptor, B7-3, identified by a monoclonal antibody BB-1, which also binds B7-1 (Yokochi, T., et al. (1982) *J. Immunol.* 128, 823-827). The B7-3 antigen is also expressed on B7-1 negative activated B cells and can costimulate T cell proliferation without detectable IL-2 production, indicating that the B7-1 and B7-3 molecules are distinct. B7-3 is expressed on a wide variety of cells including activated B cells, activated monocytes, dendritic cells, 20 Langerhan cells and keratinocytes. At 72 hours post B cell activation, the expression of B7-1 and B7-3 begins to decline. The presence of these costimulatory molecules on the surface of activated B lymphocytes indicates that T cell costimulation is regulated, in part, by the temporal expression of these molecules following B cell activation.

Accordingly, one aspect of this invention pertains to isolated nucleic acids comprising 25 a nucleotide sequence encoding a novel costimulatory molecule, such as the B lymphocyte antigen, B7-2, fragments of such nucleic acids, or equivalents thereof. The term "nucleic acid" as used herein is intended to include such fragments or equivalents. The term "equivalent" is intended to include nucleotide sequences encoding functionally equivalent B lymphocyte antigens or functionally equivalent peptides having an activity of a novel B 30 lymphocyte antigen, i.e., the ability to bind to the natural ligand(s) of the B lymphocyte antigen on immune cells, such as CTLA4 and/or CD28 on T cells, and inhibit (e.g., block) or stimulate (e.g., enhance) immune cell costimulation. Such nucleic acids are considered equivalents of the human B7-2 nucleotide sequence provided in Figure 8 (SEQ ID NO:1) and the murine B7-2 nucleotide sequence provided in Figure 14 (SEQ ID NO:22) and are within 35 the scope of this invention.

In one embodiment, the nucleic acid is a cDNA encoding a peptide having an activity of the B7-2 B lymphocyte antigen. Preferably, the nucleic acid is a cDNA molecule

-11-

consisting of at least a portion of a nucleotide sequence encoding human B7-2, as shown in Figure 8 (SEQ ID NO:1) or at least a portion of a nucleotide sequence encoding murine B7-2, as shown in Figure 14 (SEQ ID NO:22). A preferred portion of the cDNA molecule of Figure 8 (SEQ ID NO:1) or Figure 14 (SEQ ID NO:22) includes the coding region of the
5 molecule.

In another embodiment, the nucleic acid of the invention encodes a peptide having an activity of B7-2 and comprising an amino acid sequence shown in Figure 8 (SEQ ID NO:2) or Figure 14 (SEQ ID NO:23). Preferred nucleic acids encode a peptide having B7-2 activity and at least about 50% homology, more preferably at least about 60% homology and most
10 preferably at least about 70% homology with an amino acid sequence shown in Figure 8 (SEQ ID NO:2). Nucleic acids which encode peptides having B7-2 activity and at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homologous with a sequence set forth in Figure 8 (SEQ ID NO:2) are also within the scope of the invention. Homology refers to sequence similarity between two peptides having the
15 activity of a novel B lymphocyte antigen, such as B7-2, or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequences is occupied by the same nucleotide base or amino acid, then the molecules are homologous at that position. A degree (or percentage) of homology between sequences is a function of the
20 number of matching or homologous positions shared by the sequences.

Another aspect of the invention provides a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid which encodes a peptide having all or a portion of an amino acid sequence shown in Figure 8 (SEQ ID NO:2) or a peptide having all or a portion of an amino acid sequence shown in Figure 14 (SEQ ID NO:23). Appropriate
25 stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high
30 stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C to high stringency conditions, at about 65°C.

Isolated nucleic acids encoding a peptide having an activity of a novel B lymphocyte antigen, as described herein, and having a sequence which differs from nucleotide sequence
35 shown in Figure 8 (SEQ ID NO:1) or Figure 14 (SEQ ID NO:22) due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (e.g., a peptide having B7-2 activity) but differ in sequence

-12-

from the sequence of Figure 8 or Figure 14 due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may occur due to degeneracy in the genetic code. As one example, DNA sequence 5 polymorphisms within the nucleotide sequence of a B7-2 (especially those within the third base of a codon) may result in "silent" mutations in the DNA which do not affect the amino acid encoded. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the B7-2 antigen will exist within a population. It will be appreciated by one skilled in the art that these variations in one or more nucleotides 10 (up to about 3-4% of the nucleotides) of the nucleic acids encoding peptides having the activity of a novel B lymphocyte antigen may exist among individuals within a population due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of the invention. Furthermore, there may be one or more isoforms or related, cross-reacting family members of the novel B lymphocyte antigens 15 described herein. Such isoforms or family members are defined as proteins related in function and amino acid sequence to a B lymphocyte antigen (e.g., the B7-2 antigen), but encoded by genes at different loci.

A "fragment" of a nucleic acid encoding a novel B lymphocyte antigen is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the 20 entire amino acid sequence of the B lymphocyte antigen and which encodes a peptide having an activity of the B lymphocyte antigen (i.e., the ability to bind to the natural ligand(s) of the B lymphocyte antigen on immune cells, such as CTLA4 and/or CD28 on T cells and either stimulate or inhibit immune cell costimulation). Thus, a peptide having B7-2 activity binds CTLA4 and/or CD28 and stimulates or inhibits a T cell mediated immune response, as 25 evidenced by, for example, cytokine production and/or T cell proliferation by T cells that have received a primary activation signal. In one embodiment, the nucleic acid fragment encodes a peptide of the B7-2 antigen which retains the ability of the antigen to bind CTLA4 and/or CD28 and deliver a costimulatory signal to T lymphocytes. In another embodiment, the nucleic acid fragment encodes a peptide including an extracellular portion of the human 30 B7-2 antigen (e.g., approximately amino acid residues 24-245 of the sequence provided in Figure 8 (SEQ ID NO:2)) which can be used to bind CTLA4 and/or CD28 and, in monovalent form, inhibit costimulation, or in multivalent form, induce or enhance costimulation.

Preferred nucleic acid fragments encode peptides of at least 20 amino acid residues in 35 length, preferably at least 40 amino acid residues and length, and more preferably at least 60 amino acid residues in length. Nucleic acid fragments which encode peptides of at least 80 amino acid residues in length, at least 100 amino acid residues in length, and at least 200 or

-13-

more amino acids in length are also within the scope of the invention. Particularly preferred nucleic acid fragments encode a peptide having the activity of human B7-2 and an amino acid sequence represented by a formula:

5



In the formula, Y comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2). X_n and Z_m are additional amino acid residue(s) linked to Y by an amide bond. X_n and Z_m are selected from amino acid residues contiguous to Y in the amino acid sequence shown in Figure 8 (SEQ ID NO:2). In the formula, X_n is amino acid residue(s) selected from amino acids contiguous to the amino terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2), i.e., from amino acid residue 23 to 1. Z_m is amino acid residue(s) selected from amino acids contiguous to the carboxy terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2), i.e., from amino acid residue 246 to 329. In addition, in the formula, n is a number from 0 to 23 (n=0-23) and m is a number from 0 to 84 (m=0-84). A particularly preferred peptide has an amino acid sequence represented by the formula X_n-Y-Z_m as above, where n=0 and m=0.

Nucleic acid fragments within the scope of the invention include those capable of hybridizing with nucleic acid from other animal species for use in screening protocols to detect novel proteins that are cross-reactive with the B lymphocyte antigens described herein. These and other fragments are described in detail herein. Generally, the nucleic acid encoding a fragment of a B lymphocyte antigen will be selected from the bases coding for the mature protein, however, in some instances it may be desirable to select all or part of a fragment or fragments from the leader sequence or non-coding portion of a nucleotide sequence. Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of recombinant protein or fragments thereof. These and other modifications of nucleic acid sequences are described in further detail herein.

A nucleic acid encoding a peptide having an activity of a novel B lymphocyte antigen, such as the B7-2 antigen, may be obtained from mRNA present in activated B lymphocytes. It should also be possible to obtain nucleic acid sequences encoding B lymphocyte antigens from B cell genomic DNA. For example, the gene encoding the B7-2 antigen can be cloned from either a cDNA or a genomic library in accordance with protocols herein described. A cDNA encoding the B7-2 antigen can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNAs can then prepared from the total mRNA. Subsequently, the cDNAs can be inserted into a suitable plasmid or viral (e.g., bacteriophage) vector using any one of a number of known techniques. Genes encoding novel B lymphocyte

-14-

antigens can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided by the invention. The nucleic acids of the invention can be DNA or RNA. A preferred nucleic acid is a cDNA encoding the human B7-2 antigen having the sequence depicted in Figure 8 (SEQ ID NO:1). Another 5 preferred nucleic acid is a cDNA encoding the murine B7-2 antigen having the sequence shown on Figure 14 (SEQ ID NO:22).

This invention further pertains to expression vectors containing a nucleic acid encoding at least one peptide having the activity of a novel B lymphocyte antigen, as described herein, operably linked to at least one regulatory sequence. "Operably linked" is 10 intended to mean that the nucleotide acid sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence (e.g., in cis or trans). Regulatory sequences are art-recognized and are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are known to 15 those skilled in the art or one described in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transfected and/or the type of protein desired to be expressed. In one embodiment, the expression vector includes a nucleic acid encoding at least a portion of the B7-2 protein, such 20 as an extracellular domain portion. In another embodiment, the expression vector includes a DNA encoding a peptide having an activity of the B7-2 antigen and a DNA encoding a peptide having an activity of another B lymphocyte antigen, such as B7-1. cDNAs encoding the human B7-1 and mouse B7-1 antigens are shown in SEQ ID NO:28 and SEQ ID NO:30, respectively. The deduced amino acid sequences of these antigens are also shown in SEQ ID 25 NO:29 and SEQ ID NO:31, respectively. Such expression vectors can be used to transfect cells to thereby produce proteins or peptides, including fusion proteins or peptides encoded by nucleic acid sequences as described herein. These and other embodiments are described in further detail herein.

The invention also features methods of producing peptides having an activity of a 30 novel B lymphocyte antigen. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding a peptide having an activity of the B7-2 protein can be cultured in a medium under appropriate conditions to allow expression of the peptide to occur. In addition, one or more expression vectors containing DNA encoding a peptide having an activity of B7-2 and DNA encoding another peptide, such as a peptide 35 having an activity of a second B lymphocyte antigen (e.g., B7-1, B7-3) can be used to transfect a host cell to coexpress these peptides or produce fusion proteins or peptides. In one embodiment, a recombinant expression vector containing DNA encoding a B7-2 fusion

-15-

- protein is produced. A B7-2 fusion protein can be produced by recombinant expression of a nucleotide sequence encoding a first peptide having B7-2 activity and a nucleotide sequence encoding second peptide corresponding to a moiety that alters the solubility, affinity, stability or valency of the first peptide, for example, an immunoglobulin constant region. Preferably,
- 5 the first peptide consists of a portion of the extracellular domain of the human B7-2 antigen (e.g., approximately amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2)). The second peptide can include an immunoglobulin constant region, for example, a human C_y1 domain or C_y4 domain (e.g., the hinge, CH2 and CH3 regions of human IgC_y1, or human IgC_y4, see e.g., Capon et al. US 5,116,964, incorporated herein by reference). A
- 10 resulting B7-2Ig fusion protein may have altered B7-2 solubility, binding affinity, stability and/or valency (i.e., the number of binding sites available per molecule) and may increase the efficiency of protein purification. Fusion proteins and peptides produced by recombinant technique may be secreted and isolated from a mixture of cells and medium containing the protein or peptide. Alternatively, the protein or peptide may be retained cytoplasmically and
- 15 the cells harvested, lysed and the protein isolated. A cell culture typically includes host cells, media and other byproducts. Suitable mediums for cell culture are well known in the art. Protein and peptides can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins and peptides. Techniques for transfecting host cells and purifying proteins and peptides are described in further detail herein.
- 20 Particularly preferred human B7-2Ig fusion proteins include the extracellular domain portion or variable region-like domain of human B7-2 coupled to an immunoglobulin constant region. The immunoglobulin constant region may contain genetic modifications which reduce or eliminate effector activity inherent in the immunoglobulin structure. For example, DNA encoding the extracellular portion of human B7-2 (hB7-2), as well as DNA
- 25 encoding the variable region-like domain of human B7-2 (hB7.2V) or the constant region-like domain of human B7-2 (hB7.2C) can be joined to DNA encoding the hinge, CH2 and CH3 regions of human IgC_y1 and/or IgC_y4 modified by site directed mutagenesis. The preparation and characterization of these fusion proteins is described in detail in Example 7.
- Transfected cells which express peptides having an activity of one or more B
- 30 lymphocyte antigens (e.g., B7-2, B7-3) on the surface of the cell are also within the scope of this invention. In one embodiment, a host cell such as a COS cell is transfected with an expression vector directing the expression of a peptide having B7-2 activity on the surface of the cell. Such a transfected host cell can be used in methods of identifying molecules which inhibit binding of B7-2 to its counter-receptor on T cells or which interfere with intracellular
- 35 signaling of costimulation to T cells in response to B7-2 interaction. In another embodiment, a tumor cell such as a sarcoma, a melanoma, a leukemia, a lymphoma, a carcinoma or a neuroblastoma is transfected with an expression vector directing the expression of at least one

-16-

peptide having the activity of a novel B lymphocyte antigen on the surface of the tumor cell. In some instances, it may be beneficial to transfet a tumor cell to coexpress major histocompatibility complex (MHC) proteins, for example MHC class II α and β chain proteins or an MHC class I α chain protein, and, if necessary, a $\beta 2$ microglobulin protein.

- 5 Such transfected tumor cells can be used to induce tumor immunity in a subject. These and other embodiments are described in further detail herein.

The nucleic acid sequences of the invention can also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully

- 10 automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071, incorporated by reference herein).

Another aspect of the invention pertains to isolated peptides having an activity of a novel B lymphocyte antigen (e.g., B7-2, B7-3). A peptide having an activity of a B

- 15 lymphocyte antigen may differ in amino acid sequence from the B lymphocyte antigen, such as the human B7-2 sequence depicted in Figure 8 (SEQ ID NO:2), or murine B7-2 sequence depicted in Figure 14 (SEQ ID NO:22), but such differences result in a peptide which functions in the same or similar manner as the B lymphocyte antigen or which has the same or similar characteristics of the B lymphocyte antigen. For example, a peptide having an
20 activity of the B7-2 protein is defined herein as a peptide having the ability to bind to the natural ligand(s) of the B7-2 protein on immune cells, such as CTLA4 and/or CD28 on T cells and either stimulate or inhibit immune cell costimulation. Thus, a peptide having B7-2 activity binds CTLA4 and/or CD28 and stimulates or inhibits a T cell mediated immune response (as evidenced by, for example, cytokine production and/or proliferation by T cells
25 that have received a primary activation signal). One embodiment provides a peptide having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to T cells.

- Such a peptide can be used to inhibit or block T cell proliferation and/or cytokine secretion in a subject. Alternatively, a peptide having both B7-2 binding activity and the ability to deliver a costimulatory signal to T cells is used to stimulate or enhance T cell proliferation and/or
30 cytokine secretion in a subject. Various modifications of the B7-2 protein to produce these and other functionally equivalent peptides are described in detail herein. The term "peptide" as used herein, refers to peptides, proteins and polypeptides.

- A peptide can be produced by modification of the amino acid sequence of the human B7-2 protein shown in Figure 8 (SEQ ID NO:2) or the murine B7-2 protein shown in Figure
35 14 (SEQ ID NO:23), such as a substitution, addition or deletion of an amino acid residue which is not directly involved in the function of B7-2 (i.e., the ability of B7-2 to bind CTLA4 and/or CD28 and/or stimulate or inhibit T cell costimulation). Peptides of the invention are

-17-

typically at least 20 amino acid residues in length, preferably at least 40 amino acid residues in length, and most preferably 60 amino acid residues in length. Peptides having B7-2 activity and including at least 80 amino acid residues in length, at least 100 amino acid residues in length, or at least 200 or more amino acid residues in length are also within the 5 scope of the invention. A preferred peptide includes an extracellular domain portion of the human B7-2 antigen (e.g., about amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2). Other preferred peptides have an amino acid sequence represented by a formula:

10 X_n-Y-Z_m

where Y is amino acid residues selected from the group consisting of: amino acid residues 55-68 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 81-89 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 128-142 of the sequence 15 shown in Figure 8 (SEQ ID NO:2); amino acid residues 160-169 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 188-200 of the sequence shown in Figure 8 (SEQ ID NO:2); and amino acid residues 269-282 of the sequence shown in Figure 8 (SEQ ID NO:2). In the formula, X_n and Z_m are additional amino acid residues linked to Y by an amide bond. X_n and Z_m are amino acid residues selected from amino acids contiguous to Y 20 in the amino acid sequence shown in Figure 8 (SEQ ID NO:2). X_n is amino acid residues selected from amino acids contiguous to the amino terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2). Z_m is amino acid residues selected from amino acids contiguous to the carboxy terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2). According to 25 the formula, n is a number from 0 to 30 (n=0-30) and m is a number from 0 to 30 (m=0-30). A particularly preferred peptide has an amino acid sequence represented by the formula X_n-Y-Z_m , where n=0 and m=0.

Another embodiment of the invention provides a substantially pure preparation of a peptide having an activity of a novel B lymphocyte antigen such as B7-2 or B7-3. Such a preparation is substantially free of proteins and peptides with which the peptide naturally 30 occurs in a cell or with which it naturally occurs when secreted by a cell.

The term "isolated" as used throughout this application refers to a nucleic acid, protein or peptide having an activity of a novel B lymphocyte antigen, such as B7-2, substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. 35 An isolated nucleic acid is also free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the organism from which the nucleic acid is derived.

-18-

These and other aspects of this invention are described in detail in the following subsections.

I. Isolation of Nucleic Acid From Cell Lines

- 5 Suitable cells for use in isolating nucleic acids encoding peptides having an activity of a novel B lymphocyte antigen include cells capable of producing mRNA coding for B lymphocyte antigens (e.g., B7-1, B7-2, B7-3) and appropriately translating the mRNA into the corresponding protein. One source of mRNA is normal human splenic B cells, either resting or activated by treatment with an anti-immunoglobulin antibody or an anti-MHC class II antibody, or from subsets of neoplastic B cells. Expression of the human B7-2 antigen is detectable in resting B cells and in activated B cells, with mRNA levels increasing 4-fold from resting levels following stimulation. Total cellular RNA can be obtained using standard techniques from resting or activated B cells during these intervals and utilized in the construction of a cDNA library.
- 10 In addition, various subsets of neoplastic B cells may express B7-2 and B7-3 and can alternatively serve as a source of the mRNA for construction of a cDNA library. For example, tumor cells isolated from patients with non-Hodgkins lymphoma express B7-1 mRNA. B cells from nodular, poorly differentiated lymphoma (NPDL), diffuse large cell lymphoma (LCL) and Burkitt's lymphoma cell lines are also suitable sources of human B7-1 mRNA and, potentially B7-2 and B7-3 mRNA. Myelomas generally express B7-2, but not B7-1 mRNA, and, thus can provide a source of B7-2 mRNA. The Burkitt's lymphoma cell line Raji is one source of B lymphocyte antigen mRNA. Preferably, B7-2 mRNA is obtained from a population of both resting and activated normal human B cells. Activated B cells can be obtained by stimulation over a broad spectrum of time (e.g., from minutes to days) with, for example, an anti-immunoglobulin antibody or an anti-MCH class II antibody.
- 15
- 20
- 25

II. Isolation of mRNA and Construction of cDNA Library

- Total cellular mRNA can be isolated by a variety of techniques, e.g., by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., *Biochemistry* 18, 5294-5299 (1979). According to this method, Poly (A⁺) mRNA is prepared and purified for use in a cDNA library construction using oligo (dT) cellulose selection. cDNA is then synthesized from the poly(A⁺) RNA using oligo(dT) priming and reverse transcriptase. Moloney MLV reverse transcriptase (available from Gibco/BRL, Bethesda, MD) or AMV reverse transcriptase (available from Seikagaku America, Inc., St. Petersburg, FL) are preferably employed.

Following reverse transcription, the mRNA/DNA hybrid molecule is converted to double stranded DNA using conventional techniques and incorporated into a suitable vector.

-19-

The experiments herein employed *E. coli* DNA polymerase I and ribonuclease H in the conversion to double stranded cDNA.

Cloning of the cDNAs can be accomplished using any of the conventional techniques for joining double stranded DNA with an appropriate vector. The use of synthetic adaptors is particularly preferred, since it alleviates the possibility of cleavage of the cDNA with restriction enzyme prior to cloning. Using this method, non-self complementary, kinased adaptors are added to the DNA prior to ligation with the vector. Virtually any adaptor can be employed. As set forth in more detail in the examples below, non-self complementary BstXI adaptors are preferably added to the cDNA for cloning, for ligation into a pCDM8 vector prepared for cloning by digestion with BstXI.

Eucaryotic cDNA can be expressed when placed in the sense orientation in a vector that supplies an appropriate eucaryotic promoter and origin of replication and other elements including enhancers, splice acceptors and/or donor sequences and polyadenylation signals. The cDNAs of the present invention are placed in suitable vectors containing a eucaryotic promoter, an origin of replication functional in *E. coli*, an SV40 origin of replication which allows growth in COS cells, and a cDNA insertion site. Suitable vectors include π H3 (Seed and Aruffo, *Proc. Natl. Acad. Sci.*, 84:3365-3369 (1987)), π H3m (Aruffo and Seed, *Proc. Natl. Acad. Sci.*, 84:8573-8577 (1987)), pCDM7 and pCDM8 (Seed, *Nature*, 329:840-841 (1987), with the pCDM8 vector being particularly preferred (available commercially from Invitrogen, San Diego, CA).

III. Transfection of Host Cells and Screening for Novel B Lymphocyte Activation Antigens

The thus prepared cDNA library is then used to clone the gene of interest by expression cloning techniques. A basic expression cloning technique has been described by Seed and Aruffo, *Proc. Natl. Acad. Sci. USA*, 84:3365-3369 (1987) and Aruffo and Seed, *Proc. Natl. Acad. Sci. USA*, 84:8573-8577 (1987), although modifications to this technique may be necessary.

According to one embodiment, plasmid DNA is introduced into a simian COS cell line (Gluzman, *Cell* 23:175 (1981)) by known methods of transfection (e.g., DEAE-Dextran) and allowed to replicate and express the cDNA inserts. The transfectants expressing B7-1 antigen are depleted with an anti-B7-1 monoclonal antibody (e.g., 133 and B1.1) and anti-murine IgG and IgM coated immunomagnetic beads. Transfectants expressing human B7-2 antigen can be positively selected by reacting the transfectants with the fusion proteins CTLA4Ig and CD28Ig, followed by panning with anti-human Ig antibody coated plates. Although human CTLA4Ig and CD28Ig fusion proteins were used in the examples described herein, given the cross-species reactivity between B7-1 and, for example murine B7-1, it can be expected that other fusion proteins reactive with another cross-reactive species could be

-20-

used. After panning, episomal DNA is recovered from the panned cells and transformed into a competent bacterial host, preferably *E. coli*. Plasmid DNA is subsequently reintroduced into COS cells and the cycle of expression and panning repeated at least two times. After the final cycle, plasmid DNA is prepared from individual colonies, transfected into COS cells
5 and analyzed for expression of novel B lymphocyte antigens by indirect immunofluorescence with, for example, CTLA4Ig and CD28Ig.

IV. Sequencing of Novel B Lymphocyte Antigens

Plasmids are prepared from those clones which are strongly reactive with the
10 CTLA4Ig and/or CD28Ig. These plasmids are then sequenced. Any of the conventional sequencing techniques suitable for sequencing tracts of DNA about 1.0 kb or larger can be employed.

As described in Example 4, a human B7-2 clone (clone29) was obtained containing an insert of 1,120 base pairs with a single long open reading frame of 987 nucleotides and
15 approximately 27 nucleotides of 3' noncoding sequences (Figure 8, SEQ ID NO:1). The predicted amino acid sequence encoded by the open reading frame of the protein is shown below the nucleotide sequence in Figure 8. The encoded human B7-2 protein, is predicted to be 329 amino acid residues in length (SEQ ID NO:2). This protein sequence exhibits many features common to other type I Ig superfamily membrane proteins. Protein translation is
20 predicted to begin at the methionine codon (ATG, nucleotides 107 to 109) based on the DNA homology in this region with the consensus eucaryotic translation initiation site (see Kozak, M. (1987) *Nucl. Acids Res.* **15**:8125-8148). The amino terminus of the B7-2 protein (amino acids 1 to 23) has the characteristics of a secretory signal peptide with a predicted cleavage between the alanines at positions 23 and 24 (von Heijne (1987) *Nucl. Acids Res.* **14**:4683).
25 Processing at this site would result in a B7-2 membrane bound protein of 306 amino acids having an unmodified molecular weight of approximately 34 kDa. This protein would consist of an approximate extracellular Ig superfamily V and C like domains of from about amino acid residue 24 to 245, a hydrophobic transmembrane domain of from about amino acid residue 246 to 268, and a long cytoplasmic domain of from about amino acid residue
30 269 to 329. The homologies to the Ig superfamily are due to the two contiguous Ig-like domains in the extracellular region bound by the cysteines at positions 40 to 110 and 157 to 218. The extracellular domain also contains eight potential N-linked glycosylation sites and, like B7-1, is probably glycosylated. Glycosylation of the human B7-2 protein may increase the molecular weight to about 50-70 kDa. The cytoplasmic domain of human B7-2, while
35 somewhat longer than B7-1, contains a common region of multiple cysteines followed by positively charged amino acids which presumably function as signaling or regulatory domains within an antigen-presenting cell (APC). Comparison of both the nucleotide and

-21-

amino acid sequences of the human B7-2 with the GenBank and EMBL databases yielded significant homology (about 26% amino acid sequence identity) with human B7-1. Since human B7-1, human B7-2 and murine B7-1 all bind to human CTLA4 and CD28, the homologous amino acids probably represent those necessary to comprise a CTLA4 or CD28 binding sequence. *E. coli* transfected with a vector containing a cDNA insert encoding human B7-2 (clone 29) was deposited with the American Type Culture Collection (ATCC) on July 26, 1993 as Accession No. 69357.

V. Cloning Novel B Lymphocyte Antigens from Other Mammalian Species

10 The present invention is not limited to human nucleic acid molecules and contemplates that novel B lymphocyte antigen homologues from other mammalian species that express B lymphocyte antigens can be cloned and sequenced using the techniques described herein. B lymphocyte antigens isolated for one species (e.g., humans) which exhibit cross-species reactivity may be used to modify T cell mediated immune responses in a 15 different species (e.g., mice). Isolation of cDNA clones from other species can also be accomplished using human cDNA inserts, such as human B7-2 cDNA, as hybridization probes.

As described in Example 6, a murine B7-2 clone (mB7-2, clone 4) was obtained containing an insert of 1,163 base pairs with a single long open reading frame of 927 20 nucleotides and approximately 126 nucleotides of 3' noncoding sequences (Figure 14, SEQ ID NO:22). The predicted amino acid sequence encoded by the open reading frame of the protein is shown below the nucleotide sequence in Figure 14. The encoded murine B7-2 protein, is predicted to be 309 amino acid residues in length (SEQ ID NO:23). This protein sequence exhibits many features common to other type I Ig superfamily membrane proteins. 25 Protein translation is predicted to begin at the methionine codon (ATG, nucleotides 111 to 113) based on the DNA homology in this region with the consensus eucaryotic translation initiation site (see Kozak, M. (1987) *Nucl. Acids Res.* **15**:8125-8148). The amino terminus of the murine B7-2 protein (amino acids 1 to 23) has the characteristics of a secretory signal peptide with a predicted cleavage between the alanine at position 23 and the valine at position 30 24 (von Heijne (1987) *Nucl. Acids Res.* **14**:4683). Processing at this site would result in a murine B7-2 membrane bound protein of 286 amino acids having an unmodified molecular weight of approximately 32 kDa. This protein would consist of an approximate extracellular Ig superfamily V and C like domains of from about amino acid residue 24 to 246, a hydrophobic transmembrane domain of from about amino acid residue 247 to 265, and a long 35 cytoplasmic domain of from about amino acid residue 266 to 309. The homologies to the Ig superfamily are due to the two contiguous Ig-like domains in the extracellular region bound by the cysteines at positions 40 to 110 and 157 to 216. The extracellular domain also

-22-

- contains nine potential N-linked glycosylation sites and, like murine B7-1, is probably glycosylated. Glycosylation of the murine B7-2 protein may increase the molecular weight to about 50-70 kDa. The cytoplasmic domain of murine B7-2 contains a common region which has a cysteine followed by positively charged amino acids which presumably functions as
- 5 signaling or regulatory domain within an APC. Comparison of both the nucleotide and amino acid sequences of murine B7-2 with the GenBank and EMBL databases yielded significant homology (about 26% amino acid sequence identity) with human and murine B7-1. Murine B7-2 exhibits about 50% identity and 67% similarity with its human homologue, hB7-2. *E. coli* (DH106/p3) transfected with a vector (plasmid pmBx4) containing a cDNA
- 10 insert encoding murine B7-2 (clone 4) was deposited with the American Type Culture Collection (ATCC) on August 18, 1993 as Accession No. 69388.

Nucleic acids which encode novel B lymphocyte antigens from other species, such as the murine B7-2, can be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents.

15 A transgenic animal (e.g., a mouse) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, murine B7-2 cDNA or an appropriate sequence thereof can be used to clone genomic B7-2 in accordance with

20 established techniques and the genomic sequences used to generate transgenic animals that contain cells which express B7-2 protein. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for B7-2 transgene incorporation with tissue specific enhancers, which could result

25 in T cell costimulation and enhanced T cell proliferation and autoimmunity. Transgenic animals that include a copy of a B7-2 transgene introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased B7 expression. Such animals can be used as tester animals for reagents thought to confer protection from, for example, autoimmune disease. In accordance with this facet of the invention, an animal is

30 treated with the reagent and a reduced incidence of the disease, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the disease.

Alternatively, the non-human homologues of B7-2 can be used to construct a B7-2 "knock out" animal which has a defective or altered B7-2 gene as a result of homologous recombination between the endogenous B7-2 gene and altered B7-2 genomic DNA introduced into an embryonic cell of the animal. For example, murine B7-2 cDNA can be used to clone genomic B7-2 in accordance with established techniques. A portion of the

-23-

genomic B7-2 DNA (e.g., such as an exon which encodes an extracellular domain) can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas, K.R. and Capecchi, M. R. 5 (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see e.g., Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, A. in 10 *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harbouring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal 15 contain the homologously recombined DNA. Knockout animals can be characterized for their ability to accept grafts, reject tumors and defend against infectious diseases and can be used in the study of basic immunobiology.

VI. Expression of B Lymphocyte Antigens

20 Host cells transfected to express peptides having the activity of a novel B lymphocyte antigen are also within the scope of the invention. The host cell may be any prokaryotic or eucaryotic cell. For example, a peptide having B7-2 activity may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) and NS0 cells. Other suitable host cells may be found in 25 Goeddel, (1990) *supra* or are known to those skilled in the art.

For example, expression in eucaryotic cells such as mammalian, yeast, or insect cells can lead to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of recombinant protein. Examples of vectors for expression in yeast *S. cerevisiae* include pYEpSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and 30 pYES2 (Invitrogen Corporation, San Diego, CA). Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39). Generally, COS cells (Gluzman, Y., (1981) *Cell* 23:175- 35 182) are used in conjunction with such vectors as pCDM8 (Seed, B., (1987) *Nature* 329:840) for transient amplification/expression in mammalian cells, while CHO (dhfr⁻ Chinese Hamster Ovary) cells are used with vectors such as pMT2PC (Kaufman et al. (1987),

-24-

EMBO J. 6:187-195) for stable amplification/expression in mammalian cells. A preferred cell line for production of recombinant protein is the NS0 myeloma cell line available from the ECACC (catalog #85110503) and described in Galfre, G. and Milstein, C. ((1981) *Methods in Enzymology* 73(13):3-46; and *Preparation of Monoclonal Antibodies: Strategies and Procedures*, Academic Press, N.Y., N.Y.). Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, or electroporation. Suitable methods for transforming host cells can be found in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks. When used in mammalian cells, the expression vector's control functions are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and most frequently, Simian Virus 40.

It is known that a small fraction of cells (about 1 out of 10^5) typically integrate DNA into their genomes. In order to identify these integrants, a gene that contains a selectable marker (i.e., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Selectable markers may be introduced on the same plasmid as the gene of interest or may be introduced on a separate plasmid. Cells containing the gene of interest can be identified by drug selection; cells that have incorporated the selectable marker gene will survive, while the other cells die. The surviving cells can then be screened for production of novel B lymphocyte antigens by cell surface staining with ligands to the B cell antigens (e.g., CTLA4Ig and CD28Ig). Alternatively, the protein can be metabolically radiolabeled with a labeled amino acid and immunoprecipitated from cell supernatant with an anti-B lymphocyte antigen monoclonal antibody or a fusion protein such as CTLA4Ig or CD28Ig.

Expression in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids usually to the amino terminus of the expressed target gene. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the target recombinant protein; and 3) to aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the target recombinant protein to enable separation of the target recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Amrad

-25-

Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRITS (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E binding protein, or protein A, respectively, to the target recombinant protein.

E. coli expression systems include the inducible expression vectors pTrc (Amann et al., 1988) *Gene* 69:301-315 and pET 11 (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89; commercially available from Novagen). In the pTrc vector system, the inserted gene is expressed with a pelB signal sequence by host RNA polymerase transcription from a hybrid trp-lac fusion promoter. After induction, the recombinant protein can be purified from the 10 periplasmic fraction. In the pET 11 vector system, the target gene is expressed as non-fusion protein by transcription from the T7 gn10-lac O fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host *E. coli* strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 under the transcriptional control of the lacUV 5 promoter. In this system, the recombinant protein can 15 be purified from inclusion bodies in a denatured form and, if desired, renatured by step gradient dialysis to remove denaturants.

One strategy to maximize recombinant B7-2 expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, 20 Academic Press, San Diego, California (1990) 119-128). Another strategy would be to alter the nucleic acid sequence of the B7-2 gene or other DNA to be inserted into an expression vector so that the individual codons for each amino acid would be those preferentially utilized in highly expressed *E. coli* proteins (Wada et al., (1992) *Nuc. Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention could be carried out by standard 25 DNA synthesis techniques.

Novel B lymphocyte antigens and portions thereof, expressed in mammalian cells or otherwise, can be purified according to standard procedures of the art, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, electrophoresis, affinity chromatography, etc.) and ultimately, crystallization (see generally, 30 "Enzyme Purification and Related Techniques", *Methods in Enzymology*, 22:233-577 (1971)). Once purified, partially or to homogeneity, the recombinantly produced B lymphocyte antigens or portions thereof can be utilized in compositions suitable for pharmaceutical administration as described in detail herein.

-26-

VII. Modifications of Nucleic Acid and Amino Acid Sequences of the Invention
and Assays for B7 Lymphocyte Antigen Activity

It will be appreciated by those skilled in the art that other nucleic acids encoding peptides having the activity of a novel B lymphocyte antigen can be isolated by the above process. Different cell lines can be expected to yield DNA molecules having different sequences of bases. Additionally, variations may exist due to genetic polymorphisms or cell-mediated modifications of the genetic material. Furthermore, the DNA sequence of a B lymphocyte antigen can be modified by genetic techniques to produce proteins or peptides with altered amino acid sequences. Such sequences are considered within the scope of the present invention, where the expressed peptide is capable of either inducing or inhibiting activated T cell mediated immune responses and immune function.

A number of processes can be used to generate equivalents or fragments of an isolated DNA sequence. Small subregions or fragments of the nucleic acid encoding the B7-2 protein, for example 1-30 bases in length, can be prepared by standard, synthetic organic chemical means. The technique is also useful for preparation of antisense oligonucleotides and primers for use in the generation of larger synthetic fragments of B7-2 DNA.

Larger subregions or fragments of the genes encoding B lymphocyte antigens can be expressed as peptides by synthesizing the relevant piece of DNA using the polymerase chain reaction (PCR) (Sambrook, Fritsch and Maniatis, *2 Molecular Cloning; A Laboratory Manual*, Cold Spring Harbor, N.Y., (1989)), and ligating the thus obtained DNA into an appropriate expression vector. Using PCR, specific sequences of the cloned double stranded DNA are generated, cloned into an expression vector, and then assayed for CTLA4/CD28 binding activity. For example, to express a secreted (soluble) form of the human B7-2 protein, using PCR, a DNA can be synthesized which does not encode the transmembrane and cytoplasmic regions of the protein. This DNA molecule can be ligated into an appropriate expression vector and introduced into a host cell such as CHO, where the B7-2 protein fragment is synthesized and secreted. The B7-2 protein fragment can then readily be obtained from the culture media.

In another embodiment, mutations can be introduced into a DNA by any one of a number of methods, including those for producing simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases, to generate variants or modified equivalents of B lymphocyte antigen DNA. For example, changes in the human B7-2 cDNA sequence shown in Figure 8 (SEQ ID NO:1) or murine B7-2 cDNA sequence shown in Figure 14 (SEQ ID NO:22) such as amino acid substitutions or deletions are preferably obtained by site-directed mutagenesis. Site directed mutagenesis systems are well known in the art. Protocols and reagents can be obtained commercially from Amersham International PLC, Amersham, U.K.

-27-

Peptides having an activity of a novel B lymphocyte antigen, i.e., the ability to bind to the natural ligand(s) of a B lymphocyte antigen on T cells and either stimulate (amplify) or inhibit (block) activated T cell mediated immune responses, as evidenced by, for example, cytokine production and/or T cell proliferation by T cells that have received a primary activation signal are considered within the scope of the invention. More specifically, peptides that bind to T lymphocytes, for example CD28⁺ cells, may be capable of delivering a costimulatory signal to the T lymphocytes, which, when transmitted in the presence of antigen and class II MHC, or other material capable of transmitting a primary signal to the T cell, results in activation of cytokine genes within the T cell. Alternatively, such a peptide can be used in conjunction with class I MHC to thereby activate CD8⁺ cytolytic T cells. In addition, soluble, monomeric forms of the B7-2 protein, may retain the ability to bind to their natural ligand(s) on CD28⁺ T cells but, perhaps because of insufficient cross-linking with the ligand, fail to deliver the secondary signal essential for enhanced cytokine production and cell division. Such peptides, which provide a means to induce a state of anergy or tolerance in the cells, are also considered within the scope of the invention.

Screening the peptides for those which retain a characteristic B lymphocyte antigen activity as described herein can be accomplished using one or more of several different assays. For example, the peptides can be screened for specific reactivity with an anti-B7-2 monoclonal antibody reactive with cell surface B7-2 or with a fusion protein, such as CTLA4Ig or CD28Ig. Specifically, appropriate cells, such as COS cells, can be transfected with a B7-2 DNA encoding a peptide and then analyzed for cell surface phenotype by indirect immunofluorescence and flow cytometry to determine whether the peptide has B7-2 activity. Cell surface expression of the transfected cells is evaluated using a monoclonal antibody specifically reactive with cell surface B7-2 or with a CTLA4Ig or CD28Ig fusion protein. Production of secreted forms of B7-2 is evaluated using anti-B7-2 monoclonal antibody or CTLA4Ig or CD28 fusion protein for immunoprecipitation.

Other, more preferred, assays take advantage of the functional characteristics of the B7-2 antigen. As previously set forth, the ability of T cells to synthesize cytokines depends not only on occupancy or cross-linking of the T cell receptor for antigen (the "primary activation signal" provided by, for example anti-CD3, or phorbol ester to produce an "activated T cell"), but also on the induction of a costimulatory signal, in this case, by interaction with a B lymphocyte antigen, such as B7-2, B7-1 or B7-3. The binding of B7-2 to its natural ligand(s) on, for example, CD28⁺ T cells, has the effect of transmitting a signal to the T cell that induces the production of increased levels of cytokines, particularly of interleukin-2, which in turn stimulates the proliferation of the T lymphocytes. Other assays for B7-2 function thus involve assaying for the synthesis of cytokines, such as interleukin-2,

-28-

interleukin-4 or other known or unknown novel cytokines, and/or assaying for T cell proliferation by CD28⁺ T cells which have received a primary activation signal.

In vitro, T cells can be provided with a first or primary activation signal by anti-T3 monoclonal antibody (e.g. anti-CD3) or phorbol ester or, more preferably, by antigen in association with class II MHC. T cells which have received a primary activation signal are referred to herein as activated T cells. B7-2 function is assayed by adding a source of B7-2 (e.g., cells expressing a peptide having B7-2 activity or a secreted form of B7-2) and a primary activation signal such as antigen in association with Class II MHC to a T cell culture and assaying the culture supernatant for interleukin-2, gamma interferon, or other known or unknown cytokine. For example, any one of several conventional assays for interleukin-2 can be employed, such as the assay described in *Proc. Natl. Acad. Sci. USA*, 86:1333 (1989) the pertinent portions of which are incorporated herein by reference. A kit for an assay for the production of interferon is also available from Genzyme Corporation (Cambridge, MA.). T cell proliferation can also be measured as described in the Examples below. Peptides that retain the characteristics of the B7-2 antigen as described herein may result in increased per cell production of cytokines, such as IL-2, by T cells and may also result in enhanced T cell proliferation when compared to a negative control in which a costimulatory signal is lacking.

The same basic functional assays can also be used to screen for peptides having B7-2 activity, but which lack the ability to deliver a costimulatory signal, but in the case of such peptides, addition of the B7-2 protein will not result in a marked increase in proliferation or cytokine secretion by the T cells. The ability of such proteins to inhibit or completely block the normal B7-2 costimulatory signal and induce a state of anergy can be determined using subsequent attempts at stimulation of the T cells with antigen presenting cells that express cell surface B7-2 and present antigen. If the T cells are unresponsive to the subsequent activation attempts, as determined by IL-2 synthesis and T cell proliferation, a state of anergy has been induced. See, e.g., Gimmi, C.D. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90, 6586-6590; and Schwartz (1990) *Science*, 248, 1349-1356, for assay systems that can be used as the basis for an assay in accordance with the present invention.

It is possible to modify the structure of a peptide having the activity of a novel B lymphocyte antigen for such purposes as increasing solubility, enhancing therapeutic or prophylactic efficacy, or stability (e.g., shelf life *ex vivo* and resistance to proteolytic degradation *in vivo*). Such modified peptides are considered functional equivalents of the B lymphocyte antigens as defined herein. For example, a peptide having B7-2 activity can be modified so that it maintains the ability to co-stimulate T cell proliferation and/or produce cytokines. Those residues shown to be essential to interact with the CTLA4/CD28 receptors on T cells can be modified by replacing the essential amino acid with another, preferably similar amino acid residue (a conservative substitution) whose presence is shown to enhance,

-29-

diminish, but not eliminate, or not effect receptor interaction. In addition, those amino acid residues which are not essential for receptor interaction can be modified by being replaced by another amino acid whose incorporation may enhance, diminish, or not effect reactivity.

Another example of modification of a peptide having the activity of a novel B

- 5 lymphocyte antigen is substitution of cysteine residues preferably with alanine, serine, threonine, leucine or glutamic acid residues to minimize dimerization via disulfide linkages. In addition, amino acid side chains of a peptide having B7-2 activity can be chemically modified. Another modification is cyclization of the peptide.

In order to enhance stability and/or reactivity, peptides having B7-2 activity can be
10 modified to incorporate one or more polymorphisms in the amino acid sequence of the antigen resulting from any natural allelic variation. Additionally, D-amino acids, non-natural amino acids, or non-amino acid analogs can be substituted or added to produce a modified protein within the scope of this invention. Furthermore, the peptides can be modified using polyethylene glycol (PEG) according to the method of A. Sehon and co-workers (Wie et al.,
15 supra) to produce a peptide conjugated with PEG. In addition, PEG can be added during chemical synthesis of the peptide. Other modifications of the peptides include reduction/alkylation (Tarr in: *Methods of Protein Microcharacterization*, J. E. Silver ed., Humana Press, Clifton NJ 155-194 (1986)); acylation (Tarr, supra); chemical coupling to an appropriate carrier (Mishell and Shiigi, eds, *Selected Methods in Cellular Immunology*, WH Freeman, San Francisco, CA (1980), U.S. Patent 4,939,239; or mild formalin treatment (Marsh (1971), *Int. Arch. of Allergy and Appl. Immunol.* 41:199-215)).

To facilitate purification and potentially increase solubility of a peptide, it is possible to add an amino acid fusion moiety to the protein backbone. For example, hexa-histidine can be added to the peptide for purification by immobilized metal ion affinity chromatography
25 (Hochuli, E. et al., (1988) *Bio/Technology* 6:1321-1325). In addition, to facilitate isolation of a B lymphocyte antigen free of irrelevant sequences, specific endoprotease cleavage sites can be introduced between the sequences of a fusion moiety and the peptide. It may be necessary to increase the solubility of a peptide by adding functional groups to the peptide, or by omitting hydrophobic regions of the peptide.

30

VII. Uses of Nucleic Acid Sequences Encoding B Lymphocyte Antigens and Peptides Having B7-2 Activity

A. Molecular Probes

35 The nucleic acids of this invention are useful diagnostically, for tracking the progress of disease, by measuring the activation status of B lymphocytes in biological samples or for assaying the effect of a molecule on the expression of a B lymphocyte antigen (e.g.,

-30-

detecting cellular mRNA levels). In accordance with these diagnostic assays, the nucleic acid sequences are labeled with a detectable marker, e.g., a radioactive, fluorescent, or biotinylated marker and used in a conventional dot blot or Northern hybridization procedure to probe mRNA molecules of total or poly(A+) RNAs from a biological sample.

5

B. Antibody Production

The peptides and fusion proteins produced from the nucleic acid molecules of the present invention can also be used to produce antibodies specifically reactive with B lymphocyte antigens. For example, by using a full-length B7-2 protein, or a peptide fragment 10 thereof, having an amino acid sequence based on the predicted amino acid sequence of B7-2, anti-protein/anti-peptide polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the protein or peptide which elicits an antibody response in the mammal. The immunogen can be, for example, a recombinant B7-2 protein, or fragment 15 thereof, a synthetic peptide fragment or a cell that expresses a B lymphocyte antigen on its surface. The cell can be for example, a splenic B cell or a cell transfected with a nucleic acid encoding a B lymphocyte antigen of the invention (e.g., a B7-2 cDNA) such that the B lymphocyte antigen is expressed on the cell surface. The immunogen can be modified to increase its immunogenicity. For example, techniques for conferring immunogenicity on a 20 peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen to assess the levels of antibodies.

25 Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma 30 technique originally developed by Kohler and Milstein (*Nature* (1975) 256:495-497) as well as other techniques such as the human B-cell hybridoma technique (Kozbar et al., *Immunol. Today* (1983) 4:72), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. *Monoclonal Antibodies in Cancer Therapy* (1985) (Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., *Science* (1989) 246:1275). 35 Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and monoclonal antibodies isolated.

-31-

- The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with a peptide having the activity of a novel B lymphocyte antigen or fusion protein as described herein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules having an anti-B lymphocyte antigen (i.e., B7-2, B7-3) portion.
- Particularly preferred antibodies are anti-human B7-2 monoclonal antibodies produced by hybridomas HA3.1F9, HA5.2B7 and HF2.3D1. The preparation and characterization of these antibodies is described in detail in Example 8. Monoclonal antibody HA3.1F9 was determined to be of the IgG1 isotype; monoclonal antibody HA5.2B7 was determined to be of the IgG2b isotype; and monoclonal antibody HF2.3D1 was determined to be of the IgG2a isotype. Hybridoma cells were deposited with the American Type Culture Collection, which meets the requirements of the Budapest Treaty, on July 19, 1994 as ATCC Accession No. ____ (hybridoma HA3.1F9), ATCC Accession No. ____ (HA5.2B7) and ATCC Accession No. ____ (HF2.3D1).
- When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described and can be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the gene product of the novel B lymphocyte antigens of the invention. See, for example, Morrison et al., *Proc. Natl. Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. It is expected that such chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.
- For human therapeutic purposes, the monoclonal or chimeric antibodies specifically reactive with a peptide having the activity of a B lymphocyte antigen as described herein can be further humanized by producing human variable chimeras, in which parts of the

-32-

- variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. General reviews of "humanized" chimeric antibodies are provided by Morrison, S. L. (1985) *Science* 229:1202-1207 and by Oi et al. (1986) *BioTechniques* 4:214. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today*, 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain. Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution (see U.S. Patent 5,225,539 to Winter; Jones et al. (1986) *Nature* 321:552-525; Verhoeven et al. (1988) *Science* 239:1534; and Beidler et al. (1988) *J. Immunol.* 141:4053-4060). Humanized antibodies which have reduced immunogenicity are preferred for immunotherapy in human subjects.
- 15 Immunotherapy with a humanized antibody will likely reduce the necessity for any concomitant immunosuppression and may result in increased long term effectiveness for the treatment of chronic disease situations or situations requiring repeated antibody treatments.
- As an alternative to humanizing a monoclonal antibody from a mouse or other species, a human monoclonal antibody directed against a human protein can be generated. Transgenic mice carrying human antibody repertoires have been created which can be immunized with a human B lymphocyte antigen, such as B7-2. Splenocytes from these immunized transgenic mice can then be used to create hybridomas that secrete human monoclonal antibodies specifically reactive with a human B lymphocyte antigen (see, e.g., Wood et al. PCT publication WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. PCT publication WO 92/03918; Kay et al. PCT publication 92/03917; Lonberg, N. et al. (1994) *Nature* 368:856-859; Green, L.L. et al. (1994) *Nature Genet.* 7:13-21; Morrison, S.L. et al. (1994) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. (1993) *Year Immunol.* 7:33-40; Tuallion et al. (1993) *PNAS* 90:3720-3724; and Bruggeman et al. (1991) *Eur J Immunol.* 21:1323-1326).
- 30 Monoclonal antibody compositions of the invention can also be produced by other methods well known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies that bind a B lymphocyte antigen of the invention (for descriptions of combinatorial antibody display see e.g., Sastry et al. (1989) *PNAS* 86:5728; Huse et al. (1989) *Science* 246:1275; and Orlandi et al. (1989) *PNAS* 86:3833). After immunizing an animal with a B lymphocyte antigen, the antibody repertoire

-33-

- of the resulting B-cell pool is cloned. Methods are generally known for directly obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al. (1991) *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al. (1991) *Methods: Companion to Methods in Enzymology* 2:106-110).
- 5 In an illustrative embodiment, RNA is isolated from activated B cells of, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of
- 10 the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combination, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease
- 15 recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.
- 20 In an illustrative embodiment, RNA is isolated from activated B cells of, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of
- 25 the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combination, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease
- 30 recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large diverse antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP™* phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a diverse antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et

-34-

al. (1989) *Science* **246**:1275-1281; Griffiths et al. (1993) *EMBO J* **12**:725-734; Hawkins et al. (1992) *J Mol Biol* **226**:889-896; Clackson et al. (1991) *Nature* **352**:624-628; Gram et al. (1992) *PNAS* **89**:3576-3580; Garrad et al. (1991) *Bio/Technology* **9**:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* **19**:4133-4137; and Barbas et al. (1991) *PNAS* **88**:7978-7982.

5 In certain embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) **348**:552-554, complete V_H and V_L domains of an antibody, joined by a flexible (Gly₄-Ser)₃ linker can be used to
10 produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with a peptide having activity of a B lymphocyte antigen can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

Once displayed on the surface of a display package (e.g., filamentous phage), the
15 antibody library is screened with a B lymphocyte antigen protein, or peptide fragment thereof, to identify and isolate packages that express an antibody having specificity for the B lymphocyte antigen. Nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques.

20 The antibodies of the current invention can be used therapeutically to inhibit T cell activation through blocking receptor:ligand interactions necessary for costimulation of the T cell. These so-called "blocking antibodies" can be identified by their ability to inhibit T cell proliferation and/or cytokine production when added to an *in vitro* costimulation assay as described herein. The ability of blocking antibodies to inhibit T cell functions may result in
25 immunosuppression and/or tolerance when these antibodies are administered *in vivo*.

C. Protein Purification

The polyclonal or monoclonal antibodies of the current invention, such as an antibody specifically reactive with a recombinant or synthetic peptide having B7-2 activity or B7-3
30 activity can also be used to isolate the native B lymphocyte antigen from cells. For example, antibodies reactive with the peptide can be used to isolate the naturally-occurring or native form of B7-2 from activated B lymphocytes by immunoaffinity chromatography. In addition, the native form of B7-3 can be isolated from B cells by immunoaffinity chromatography with monoclonal antibody BB-1.

D. Other Therapeutic Reagents

The nucleic acid sequences and novel B lymphocyte antigens described herein can be used in the development of therapeutic reagents having the ability to either upregulate (e.g., amplify) or downregulate (e.g., suppress or tolerize) T cell mediated immune responses. For example, peptides having B7-2 activity, including soluble, monomeric forms of the B7-2 antigen or a B7-2 fusion protein, e.g., B7-2Ig, and anti-B7-2 antibodies that fail to deliver a costimulatory signal to T cells that have received a primary activation signal, can be used to block the B7-2 ligand(s) on T cells and thereby provide a specific means by which to cause immunosuppression and/or induce tolerance in a subject. Such blocking or inhibitory forms of B lymphocyte antigens and fusion proteins and blocking antibodies can be identified by their ability to inhibit T cell proliferation and/or cytokine production when added to an *in vitro* costimulation assay as previously described herein. In contrast to the monomeric form, stimulatory forms of B7-2, such as an intact cell surface B7-2, retain the ability to transmit the costimulatory signal to the T cells, resulting in an increased secretion of cytokines when compared to activated T cells that have not received the secondary signal.

In addition, fusion proteins comprising a first peptide having an activity of B7-2 fused to a second peptide having an activity of another B lymphocyte antigen (e.g., B7-1) can be used to modify T cell mediated immune responses. Alternatively, two separate peptides having an activity of B lymphocyte antigens, for example, B7-2 and B7-1, or a combination of blocking antibodies (e.g., anti-B7-2 and anti-B7-1 monoclonal antibodies) can be combined as a single composition or administered separately (simultaneously or sequentially), to upregulate or downregulate T cell mediated immune responses in a subject. Furthermore, a therapeutically active amount of one or more peptides having B7-2 activity and or B7-1 activity can be used in conjunction with other immunomodulating reagents to influence immune responses. Examples of other immunomodulating reagents include blocking antibodies, e.g., against CD28 or CTLA4, against other T cell markers or against cytokines, fusion proteins, e.g., CTLA4Ig, or immunosuppressive drugs, e.g., cyclosporine A or FK506.

The peptides produced from the nucleic acid molecules of the present invention may also be useful in the construction of therapeutic agents which block T cell function by destruction of the T cell. For example, as described, secreted forms of a B lymphocyte antigen can be constructed by standard genetic engineering techniques. By linking a soluble form of B7-1, B7-2 or B7-3 to a toxin such as ricin, an agent capable of preventing T cell activation can be made. Infusion of one or a combination of immunotoxins, e.g., B7-2-ricin, B7-1-ricin, into a patient may result in the death of T cells, particularly of activated T cells that express higher amounts of CD28 and CTLA4. Soluble forms of B7-2 in a monovalent

-36-

form alone may be useful in blocking B7-2 function, as described above, in which case a carrier molecule may also be employed.

Another method of preventing the function of a B lymphocyte antigen is through the use of an antisense or triplex oligonucleotide. For example, an oligonucleotide 5 complementary to the area around the B7-1, B7-2 or B7-3 translation initiation site, (e.g., for B7-1, TGGCCCATGGCTTCAGA, (SEQ ID NO:20) nucleotides 326-309 and for B7-2, GCCAAATGGATCCCCA (SEQ ID NO:21)), can be synthesized. One or more antisense oligonucleotides can be added to cell media, typically at 200 µg/ml, or administered to a patient to prevent the synthesis of B7-1, B7-2 and/or B7-3. The antisense oligonucleotide is 10 taken up by cells and hybridizes to the appropriate B lymphocyte antigen mRNA to prevent translation. Alternatively, an oligonucleotide which binds double-stranded DNA to form a triplex construct to prevent DNA unwinding and transcription can be used. As a result of either, synthesis of one or more B lymphocyte antigens is blocked.

15 E. Therapeutic Uses by Downregulation of Immune Responses

Given the structure and function of the novel B lymphocyte antigens disclosed herein, it is possible to downregulate the function of a B lymphocyte antigen, and thereby downregulate immune responses, in a number of ways. Downregulation may be in the form of inhibiting or blocking an immune response already in progress or may involve preventing 20 the induction of an immune response. The functions of activated T cells may be inhibited by suppressing T cell responses or by inducing specific tolerance in T cells, or both. Immunosuppression of T cell responses is generally an active, non-antigen-specific, process which requires continuous exposure of the T cells to the suppressive agent. Tolerance, which involves inducing non-responsiveness or anergy in T cells, is distinguishable from 25 immunosuppression in that it is generally antigen-specific and persists after exposure to the tolerizing agent has ceased. Operationally, tolerance can be demonstrated by the lack of a T cell response upon reexposure to specific antigen in the absence of the tolerizing agent.

Downregulating or preventing one or more B lymphocyte antigen functions, e.g., preventing high level lymphokine synthesis by activated T cells, will be useful in situations 30 of tissue, skin and organ transplantation and in graft-versus-host disease (GVHD). For example, blockage of T cell function should result in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by T cells, followed by an immune reaction that destroys the transplant. The administration of a molecule which inhibits or blocks interaction of a B7 35 lymphocyte antigen with its natural ligand(s) on immune cells (such as a soluble, monomeric form of a peptide having B7-2 activity alone or in conjunction with a monomeric form of a peptide having an activity of another B lymphocyte antigen (e.g., B7-1, B7-3) or blocking

-37-

antibody), prior to transplantation can lead to the binding of the molecule to the natural ligand(s) on the immune cells without transmitting the corresponding costimulatory signal. Blocking B lymphocyte antigen function in this manner prevents cytokine synthesis by immune cells, such as T cells, and thus acts as an immunosuppressant. Moreover, the lack of 5 costimulation may also be sufficient to anergize the T cells, thereby inducing tolerance in a subject. Induction of long-term tolerance by B lymphocyte antigen-blocking reagents may avoid the necessity of repeated administration of these blocking reagents. To achieve sufficient immunosuppression or tolerance in a subject, it may also be necessary to block the function of a combination of B lymphocyte antigens. For example, it may be desirable to 10 block the function of B7-2 and B7-1, B7-2 and B7-3, B7-1 and B7-3 or B7-2, B7-1 and B7-3 by administering a soluble form of a combination of peptides having an activity of each of these antigens or a blocking antibody (separately or together in a single composition) prior to transplantation. Alternatively, inhibitory forms of B lymphocyte antigens can be used with other suppressive agents such as blocking antibodies against other T cell markers or against 15 cytokines, other fusion proteins, e.g., CTLA4Ig, or immunosuppressive drugs.

The efficacy of particular blocking reagents in preventing organ transplant rejection or GVHD can be assessed using animal models that are predictive of efficacy in humans. The functionally important aspects of B7-1 are conserved structurally between species and it is therefore likely that other B lymphocyte antigens can function across species, thereby 20 allowing use of reagents composed of human proteins in animal systems. Examples of appropriate systems which can be used include allogeneic cardiac grafts in rats and xenogeneic pancreatic islet cell grafts in mice, both of which have been used to examine the immunosuppressive effects of CTLA4Ig fusion proteins *in vivo* as described in Lenschow et al., *Science*, **257**: 789-792 (1992) and Turka et al., *Proc. Natl. Acad. Sci. USA*, **89**: 11102-25 11105 (1992). In addition, murine models of GVHD (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 846-847) can be used to determine the effect of blocking B lymphocyte antigen function *in vivo* on the development of that disease.

Blocking B lymphocyte antigen function, e.g., by use of a peptide having B7-2 activity alone or in combination with a peptide having B7-1 activity and/or a peptide having 30 B7-3 activity, may also be therapeutically useful for treating autoimmune diseases. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive T cells may reduce or eliminate disease symptoms. Administration of reagents which block 35 costimulation of T cells by disrupting receptor:ligand interactions of B lymphocyte antigens can be used to inhibit T cell activation and prevent production of autoantibodies or T cell-derived cytokines which may be involved in the disease process. Additionally, blocking

-38-

reagents may induce antigen-specific tolerance of autoreactive T cells which could lead to long-term relief from the disease. The efficacy of blocking reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental

- 5 autoimmunity encephalitis, systemic lupus erythematosus in MRL/lpr/lpr mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856).

The IgE antibody response in atopic allergy is highly T cell dependent and, thus, 10 inhibition of B lymphocyte antigen induced T cell activation may be useful therapeutically in the treatment of allergy and allergic reactions. An inhibitory form of B7-2 protein, such as a peptide having B7-2 activity alone or in combination with a peptide having the activity of another B lymphocyte antigen, such as B7-1, can be administered to an allergic subject to inhibit T cell mediated allergic responses in the 15 subject. Inhibition of B lymphocyte antigen costimulation of T cells may be accompanied by exposure to allergen in conjunction with appropriate MHC molecules. Allergic reactions may be systemic or local in nature, depending on the route of entry of the allergen and the pattern of deposition of IgE on mast cells or basophils. Thus, it may be necessary to inhibit T cell mediated allergic responses locally or systemically by 20 proper administration of an inhibitory form of B7-2 protein.

Inhibition of T cell activation through blockage of B lymphocyte antigen function may also be important therapeutically in viral infections of T cells. For example, in the acquired immune deficiency syndrome (AIDS), viral replication is stimulated by T cell activation. Blocking B7-2 function could lead to a lower level of viral replication and 25 thereby ameliorate the course of AIDS. In addition, it may also be necessary to block the function of a combination of B lymphocyte antigens i.e., B7-1, B7-2 and B7-3. Surprisingly, HTLV-I infected T cells express B7-1 and B7-2. This expression may be important in the growth of HTLV-I infected T cells and the blockage of B7-1 function together with the function of B7-2 and/or B7-3 may slow the growth of HTLV-I induced leukemias. 30 Alternatively, stimulation of viral replication by T cell activation may be induced by contact with a stimulatory form of B7-2 protein, for such purposes as generating retroviruses (e.g., various HIV isolates) in sufficient quantities for isolation and use.

E. Therapeutic Uses by Upregulation of Immune Responses

- 35 Upregulation of a B lymphocyte antigen function, as a means of upregulating immune responses, may also be useful in therapy. Upregulation of immune responses may be in the form of enhancing an existing immune response or eliciting an initial immune response. For

-39-

example, enhancing an immune response through stimulating B lymphocyte antigen function may be useful in cases of viral infection. Viral infections are cleared primarily by cytolytic T cells. In accordance with the present invention, it is believed that B7-2 and thus, B7-1 and B7-3 with their natural ligand(s) on T cells may result in an increase in the cytolytic activity 5 of at least some T cells. It is also believed that B7-2, B7-1, and B7-3 are involved in the initial activation and generation of CD8+ cytotoxic T cells. The addition of a soluble peptide having B7-2 activity, alone, or in combination with a peptide having the activity of another B lymphocyte antigen, in a multi-valent form, to stimulate T cell activity through the costimulation pathway would thus be therapeutically useful in situations where more rapid or 10 thorough clearance of virus would be beneficial. These would include viral skin diseases such as Herpes or shingles, in which cases the multi-valent soluble peptide having B7-2 activity or combination of such peptide and/or a peptide having B7-1 activity and/or a peptide having B7-3 activity is delivered topically to the skin. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by the 15 administration of stimulatory forms of B lymphocyte antigens systemically.

Alternatively, anti-viral immune responses may be enhanced in an infected patient by removing T cells from the patient, costimulating the T cells *in vitro* with viral antigen-pulsed APCs either expressing a peptide having B7-2 activity (alone or in combination with a peptide having B7-1 activity and/or a peptide having B7-3 activity) or together with a 20 stimulatory form of a soluble peptide having B7-2 activity (alone or in combination with a peptide having B7-1 activity and/or a peptide having B7-3 activity) and reintroducing the *in vitro* activated T cells into the patient. Another method of enhancing anti-viral immune responses would be to isolate infected cells from a patient, transfect them with a nucleic acid encoding a peptide having the activity of a B lymphocyte antigen as described herein such 25 that the cells express all or a portion of a B lymphocyte antigen on their surface, e.g., B7-2 or B7-3, and reintroduce the transfected cells into the patient. The infected cells would now be capable of delivering a costimulatory signal to, and thereby activate, T cells *in vivo*.

Stimulatory forms of B lymphocyte antigens may also be used prophylactically in vaccines against various pathogens. Immunity against a pathogen, e.g., a virus, could be 30 induced by vaccinating with a viral protein along with a stimulatory form of a peptide having B7-2 activity or another peptide having the activity of B lymphocyte antigen in an appropriate adjuvant. Alternately, an expression vector which encodes genes for both a pathogenic antigen and a peptide having the activity of a B lymphocyte antigen, e.g., a vaccinia virus expression vector engineered to express a nucleic acid encoding a viral protein 35 and a nucleic acid encoding a peptide having B7-2 activity as described herein, can be used for vaccination. Presentation of B7-2 with class I MHC proteins by, for example, a cell transfected to coexpress a peptide having B7-2 activity and MHC class I α chain protein and

-40-

β_2 microglobulin may also result in activation of cytolytic CD8+ T cells and provide immunity from viral infection. Pathogens for which vaccines may be useful include hepatitis B, hepatitis C, Epstein-Barr virus, cytomegalovirus, HIV-1, HIV-2, tuberculosis, malaria and schistosomiasis.

- 5 In another aspect, a stimulatory form of one or more soluble peptides having an activity of a B lymphocyte antigen can be administered to a tumor-bearing patient to provide a costimulatory signal to T cells in order to induce anti-tumor immunity.

G. Modification of a Tumor Cell to Express a Costimulatory Molecule

- 10 The inability of a tumor cell to trigger a costimulatory signal in T cells may be due to a lack of expression of a costimulatory molecule, failure to express a costimulatory molecule even though the tumor cell is capable of expressing such a molecule, insufficient expression of a costimulatory molecule on the tumor cell surface or lack of expression of an appropriate costimulatory molecule (e.g. expression of B7 but not B7-2 and/or B7-3). Thus, according to
15 one aspect of the invention, a tumor cell is modified to express B7-2 and/or B7-3 by transfection of the tumor cell with a nucleic acid encoding B7-2 and/or B7-3 in a form suitable for expression of B7-2 and/or B7-3 on the tumor cell surface. Alternatively, the tumor cell is modified by contact with an agent which induces or increases expression of B7-
2 and/or B7-3 on the tumor cell surface. In yet another embodiment, B7-2 and/or B7-3 is
20 coupled to the surface of the tumor cell to produce a modified tumor cell. These and other emodiments are described in further detail in the following subsections.

(1). Transfection of a Tumor Cell with a Nucleic Acid Encoding a Costimulatory Molecule

- 25 Tumor cells can be modified *ex vivo* to express B7-2 or B7-3, alone or in combination or in combination with B7-1 by transfection of isolated tumor cells with a nucleic acid encoding B7-2 and/or B7-3 and B7-1 in a form suitable for expression of the molecule on the surface of the tumor cell. The terms "transfection" or "transfected with" refers to the introduction of exogenous nucleic acid into a mammalian cell and encompass a variety of
30 techniques useful for introduction of nucleic acids into mammalian cells including electroporation, calcium-phosphate precipitation, DEAE-dextran treatment, lipofection, microinjection and infection with viral vectors. Suitable methods for transfecting mammalian cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)) and other laboratory textbooks.
35 The nucleic acid to be introduced may be, for example, DNA encompassing the gene(s) encoding B7-2 and/or B7-3, sense strand RNA encoding B7-2 and/or B7-3 or a recombinant

-41-

expression vector containing a cDNA encoding B7-2 and/or B7-3. The nucleotide sequence of a cDNA encoding human B7-2 is shown in the Sequence Listing.

A preferred approach for introducing nucleic acid encoding B7-2 and/or B7-3 into tumor cells is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding B7-2 and/or B7-3. Examples of viral vectors which can be used include retroviral vectors (Eglitis, M.A., et al., *Science* 230, 1395-1398 (1985); Danos, O. and Mulligan, R., *Proc. Natl. Acad. Sci. USA* 85, 6460-6464 (1988); Markowitz, D., et al., *J. Virol.* 62, 1120-1124 (1988)), adenoviral vectors (Rosenfeld, M.A., et al., *Cell* 68, 143-155 (1992)) and adeno-associated viral vectors (Tratschin, J.D., et al., *Mol. Cell. Biol.* 5, 3251-3260 (1985)). Infection of tumor cells with a viral vector has the advantage that a large proportion of cells will receive nucleic acid, thereby obviating a need for selection of cells which have received nucleic acid, and molecules encoded within the viral vector, e.g. by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Alternatively, B7-2 and/or B7-3 can be expressed on a tumor cell using a plasmid expression vector which contains nucleic acid, e.g. a cDNA, encoding B7-2 and/or B7-3. Suitable plasmid expression vectors include CDM8 (Seed, B., *Nature* 329, 840 (1987)) and pMT2PC (Kaufman, et al., *EMBO J.* 6, 187-195 (1987)). Suitable vectors and methods for expressing nucleic acids in host cells, such as tumor cells are described in further detail herein.

When transfection of tumor cells leads to modification of a large proportion of the tumor cells and efficient expression of B7-2 and/or B7-3 on the surface of tumor cells, e.g. when using a viral expression vector, tumor cells may be used without further isolation or subcloning. Alternatively, a homogenous population of transfected tumor cells can be prepared by isolating a single transfected tumor cell by limiting dilution cloning followed by expansion of the single tumor cell into a clonal population of cells by standard techniques.

(2). Induction or Increased Expression of a Costimulatory Molecule on a Tumor Cell Surface

A tumor cell can be modified to trigger a costimulatory signal in T cells by inducing or increasing the level of expression of B7-2 and/or B7-3 on a tumor cell which is capable of expressing B7-2 and/or B7-3 but fails to do so or which expresses insufficient amounts of B7-2 and/or B7-3 to activate T cells. An agent which stimulates expression of B7-2 and/or B7-3 can be used in order to induce or increase expression of B7-2 and/or B7-3 on the tumor cell surface. For example, tumor cells can be contacted with the agent *in vitro* in a culture medium. The agent which stimulates expression of B7-2 and/or B7-3 may act, for instance, by increasing transcription of B7-2 and/or B7-3 gene, by increasing translation of B7-2 and/or B7-3 mRNA or by increasing stability or transport of B7-2 and/or B7-3 to the cell

-42-

surface. For example, it is known that expression of B7 can be upregulated in a cell by a second messenger pathway involving cAMP. Nabavi, N., et al. *Nature* 360, 266-268 (1992). B7-2 and B7-3 may likewise be inducible by cAMP. Thus, a tumor cell can be contacted with an agent, which increases intracellular cAMP levels or which mimics cAMP, such as a 5 cAMP analogue, e.g. dibutyryl cAMP, to stimulate expression of B7-2 and/or B7-3 on the tumor cell surface. It is also known that expression of B7 can be induced on normal resting B cells by crosslinking cell-surface MHC class II molecules on the B cells with an antibody against the MHC class II molecules. Kuolova, L., et al., *J. Exp. Med.* 173, 759-762 (1991). Similarly, B7-2 and B7-3 can be induced on resting B cells by crosslinking cell-surface MHC 10 class II molecules on the B cells. Accordingly, a tumor cell which expresses MHC class II molecules on its surface can be treated with anti-MHC class II antibodies to induce or increase B7-2 and/or B7-3 expression on the tumor cell surface. In addition, interleukin-4 (IL-4) which has been found to induce expression of B7-2 on B cells, may be used to upregulate expression of B7-2 on tumor cells (Stack R.M., et al., *J. Cell. Biochem. Suppl* 15 1(18):434 (1994).

Another agent which can be used to induce or increase expression of B7-2 and/or B7-3 on a tumor cell surface is a nucleic acid encoding a transcription factor which upregulates transcription of the gene encoding the costimulatory molecule. This nucleic acid can be transfected into the tumor cell to cause increased transcription of the costimulatory molecule 20 gene, resulting in increased cell-surface levels of the costimulatory molecule.

(3). Coupling of a Costimulatory Molecule to the Surface of a Tumor Cell

In another embodiment, a tumor cell is modified to be capable of triggering a costimulatory signal in T cells by coupling B7-2 and/or B7-3 to the surface of the tumor cell. 25 For example, B7-2 and/or B7-3 molecules can be obtained using standard recombinant DNA technology and expression systems which allow for production and isolation of the costimulatory molecule(s). Alternatively, B7-2 and/or B7-3 can be isolated from cells which express the costimulatory molecule(s) using standard protein purification techniques. For example, B7-3 protein can be isolated from activated B cells by immunoprecipitation with an 30 anti-B7-3 antibody such as the BB1 monoclonal antibody. The isolated costimulatory molecule is then coupled to the tumor cell. The terms "coupled" or "coupling" refer to a chemical, enzymatic or other means (e.g., antibody) by which B7-2 and/or B7-3 is linked to a tumor cell such that the costimulatory molecule is present on the surface of the tumor cell and is capable of triggering a costimulatory signal in T cells. For example, B7-2 and/or B7-3 can 35 be chemically crosslinked to the tumor cell surface using commercially available crosslinking reagents (Pierce, Rockford IL). Another approach to coupling B7-2 and/or B7-3 to a tumor cell is to use a bispecific antibody which binds both the costimulatory molecule and a cell-

-43-

surface molecule on the tumor cell. Fragments, mutants or variants of B7-2 and/or B7-3 which retain the ability to trigger a costimulatory signal in T cells when coupled to the surface of a tumor cell can also be used.

5 (4) Modification of Tumor Cells to Express Multiple Costimulatory Molecules

Another aspect of the invention is a tumor cell modified to express multiple costimulatory molecules. The temporal expression of costimulatory molecules on activated B cells is different for B7, B7-2 and B7-3. For example, B7-2 is expressed early following B cell activation, whereas B7-3 is expressed later. The different costimulatory molecules may 10 thus serve distinct functions during the course of an immune response. An effective T cell response may require that the T cell receive costimulatory signals from multiple costimulatory molecules. Accordingly, the invention encompasses a tumor cell which is modified to express more than one costimulatory molecule. For example, a tumor cell can be modified to express both B7-2 and B7-3. Alternatively, a tumor cell modified to express B7- 15 2 can be further modified to express B7-1. Similarly, a tumor cell modified to express B7-3 can be further modified to express B7-1. A tumor cell can also be modified to express B7-1, B7-2 and B7-3. A tumor cell can be modified to express multiple costimulatory molecules (e.g., B7-1 and B7-2) by any of the techniques described herein.

Before modification, a tumor cell may not express any costimulatory molecules, or 20 may express certain costimulatory molecules but not others. As described herein, tumor cells can be modified by transfecting the tumor cell with nucleic acid encoding a costimulatory molecule(s), by inducing the expression of a costimulatory molecule(s) or by coupling a costimulatory molecule(s) to the tumor cell. For example, a tumor cell transfected with nucleic acid encoding B7-2 can be further transfected with nucleic acid encoding B7-1. The 25 cDNA sequence and deduced amino acid sequence of human B7-1 is shown in the Sequence Listing. Alternatively, more than one type of modification can be used. For example, a tumor cell transfected with a nucleic acid encoding B7-2 can be stimulated with an agent which induces expression of B7-1.

30 (5) Additional Modification of a Tumor Cell to Express MHC Molecules

Another aspect of this invention features modified tumor cells which express a costimulatory molecule and which express one or more MHC molecules on their surface to trigger both a costimulatory signal and a primary, antigen-specific, signal in T cells. Before modification, tumor cells may be unable to express MHC molecules, may fail to express 35 MHC molecules although they are capable of expressing such molecules, or may express insufficient amounts of MHC molecules on the tumor cell surface to cause T cell activation. Tumor cells can be modified to express either MHC class I or MHC class II molecules, or

-44-

both. One approach to modifying tumor cells to express MHC molecules is to transfet the tumor cell with one or more nucleic acids encoding one or more MHC molecules. Alternatively, an agent which induces or increases expression of one or more MHC molecules on tumor cells can be used to modify tumor cells. Inducing or increasing
5 expression of MHC class II molecules on a tumor cell can be particularly beneficial for activating CD4⁺ T cells against the tumor since the ability of MHC class II⁺ tumor cells to directly present tumor peptides to CD4⁺ T cells bypasses the need for professional MHC class II⁺ APCs. This can improve tumor immunogenicity because soluble tumor antigen (in the form of tumor cell debris or secreted protein) may not be available for uptake by
10 professional MHC class II⁺ APCs.

One embodiment of the invention is a modified tumor cell which expresses B7-2 and/or B7-3 and one or more MHC class II molecules on their cell surface. MHC class II molecules are cell-surface α/β heterodimers which structurally contain a cleft into which antigenic peptides bind and which function to present bound peptides to the antigen-specific
15 TcR. Multiple, different MHC class II proteins are expressed on professional APCs and different MHC class II proteins bind different antigenic peptides. Expression of multiple MHC class II molecules, therefore, increases the spectrum of antigenic peptides that can be presented by an APC or by a modified tumor cell. The α and β chains of MHC class II molecules are encoded by different genes. For instance, the human MHC class II protein
20 HLA-DR is encoded by the HLA-DR α and HLA-DR β genes. Additionally, many polymorphic alleles of MHC class II genes exist in human and other species. T cells of a particular individual respond to stimulation by antigenic peptides in conjunction with self MHC molecules, a phenomenon termed MHC restriction. In addition, certain T cells can also respond to stimulation by polymorphic alleles of MHC molecules found on the cells of other
25 individuals, a phenomenon termed allogenicity. For a review of MHC class II structure and function, see Germain and Margulies, *Ann. Rev. Immunol.* 11: 403-450, 1993.

Another embodiment of the invention is a modified tumor cell which expresses B7-2 and/or B7-3 and one or more MHC class I molecules on the cell surface. Similar to MHC class II genes, there are multiple MHC class I genes and many polymorphic alleles of these
30 genes are found in human and other species. Like MHC class II proteins, class I proteins bind peptide fragments of antigens for presentation to T cells. A functional cell-surface class I molecule is composed of an MHC class I α chain protein associated with a β 2-microglobulin protein.

35 (6). Transfection of a Tumor Cell with Nucleic Acid Encoding MHC Molecules
Tumor cells can be modified *ex vivo* to express one or more MHC class II molecules by transfection of isolated tumor cells with one or more nucleic acids encoding one or more

-45-

MHC class II α chains and one or more MHC class II β chains in a form suitable for expression of the MHC class II molecules(s) on the surface of the tumor cell. Both an α and a β chain protein must be present in the tumor cell to form a surface heterodimer and neither chain will be expressed on the cell surface alone. The nucleic acid sequences of many murine and human class II genes are known. For examples see Hood, L., et al. *Ann. Rev. Immunol.* 1, 529-568 (1983) and Auffray, C. and Strominger, J.L., *Advances in Human Genetics* 15, 197-247 (1987). Preferably, the introduced MHC class II molecule is a self MHC class II molecule. Alternatively, the MHC class II molecule could be a foreign, allogeneic, MHC class II molecule. A particular foreign MHC class II molecule to be introduced into tumor cells can be selected by its ability to induce T cells from a tumor-bearing subject to proliferate and/or secrete cytokines when stimulated by cells expressing the foreign MHC class II molecule (i.e. by its ability to induce an allogeneic response). The tumor cells to be transfected may not express MHC class II molecules on their surface prior to transfection or may express amounts insufficient to stimulate a T cell response. Alternatively, tumor cells which express MHC class II molecules prior to transfection can be further transfected with additional, different MHC class II genes or with other polymorphic alleles of MHC class II genes to increase the spectrum of antigenic fragments that the tumor cells can present to T cells.

Fragments, mutants or variants of MHC class II molecules that retain the ability to bind peptide antigens and activate T cell responses, as evidenced by proliferation and/or lymphokine production by T cells, are considered within the scope of the invention. A preferred variant is an MHC class II molecule in which the cytoplasmic domain of either one or both of the α and β chains is truncated. It is known that truncation of the cytoplasmic domains allows peptide binding by and cell surface expression of MHC class II molecules but prevents the induction of endogenous B7 expression, which is triggered by an intracellular signal generated by the cytoplasmic domains of the MHC class II protein chains upon crosslinking of cell surface MHC class II molecules. Kuolova, L., et al., *J. Exp. Med.* 173, 759-762 (1991); Nabavi, N., et al. *Nature* 360, 266-268 (1992). Expression of B7-2 and B7-3 is also induced by crosslinking surface MHC class II molecules, and thus truncation of MHC class II molecules may also prevent induction of B7-2 and/or B7-3. In tumor cells transfected to constitutively express B7-2 and/or B7-3, it may be desirable to inhibit the expression of endogenous costimulatory molecules, for instance to restrain potential downregulatory feedback mechanisms. Transfection of a tumor cell with a nucleic acid(s) encoding a cytoplasmic domain-truncated form of MHC class II α and β chain proteins would inhibit endogenous B7-1 expression and possibly also endogenous B7-2 and B7-3 expression. Such variants can be produced by, for example, introducing a stop codon in the MHC class II chain gene(s) after the nucleotides encoding the transmembrane spanning region. The cytoplasmic

-46-

domain of either the α chain or the β chain protein can be truncated, or, for more complete inhibition of B7 (and possibly B7-2 and/or B7-3) induction, both the α and β chains can be truncated. See e.g. Griffith et al., *Proc. Natl. Acad. Sci. USA* 85: 4847-4852, (1988), Nabavi et al., *J. Immunol.* 142: 1444-1447, (1989).

- 5 Tumor cells can be modified to express an MHC class I molecule by transfection with a nucleic acid encoding an MHC class I α chain protein. For examples of nucleic acids see Hood, L., et al. *Ann. Rev. Immunol.* 1, 529-568 (1983) and Auffray, C. and Strominger, J.L., *Advances in Human Genetics* 15, 197-247 (1987). Optionally, if the tumor cell does not express β -2 microglobulin, it can also be transfected with a nucleic acid encoding the β -2
10 microglobulin protein. For examples of nucleic acids see Gussow, D., et al., *J. Immunol.* 139, 3132-3138 (1987) and Parnes, J.R., et al., *Proc. Natl. Acad. Sci. USA* 78, 2253-2257 (1981). As for MHC class II molecules, increasing the number of different MHC class I genes or polymorphic alleles of MHC class I genes expressed in a tumor cell can increase the spectrum of antigenic fragments that the tumor cells can present to T cells.
15 When a tumor cell is transfected with nucleic acid which encodes more than one molecule, for example a B7-2 and/or B7-3 molecule(s), an MHC class II α chain protein and an MHC class II β chain protein, the transfections can be performed simultaneously or sequentially. If the transfections are performed simultaneously, the molecules can be introduced on the same nucleic acid, so long as the encoded sequences do not exceed a
20 carrying capacity for a particular vector used. Alternatively, the molecules can be encoded by separate nucleic acids. If the transfections are conducted sequentially and tumor cells are selected using a selectable marker, one selectable marker can be used in conjunction with the first introduced nucleic acid while a different selectable marker can be used in conjunction with the next introduced nucleic acid.
25 The expression of MHC molecules (class I or class II) on the cell surface of a tumor cell can be determined, for example, by immunofluorescence of tumor cells using fluorescently labeled monoclonal antibodies directed against different MHC molecules. Monoclonal antibodies which recognize either non-polymorphic regions of a particular MHC molecule (non-allele specific) or polymorphic regions of a particular MHC molecule (allele-specific) can be used and are known to those skilled in the art.
30

(7). Induction or Increased Expression of MHC Molecules on a Tumor Cell

Another approach to modifying a tumor cell *ex vivo* to express MHC molecules on the surface of a tumor cell is to use an agent which stimulates expression of MHC molecules in
35 order to induce or increase expression of MHC molecules on the tumor cell surface. For example, tumor cells can be contacted with the agent *in vitro* in a culture medium. An agent which stimulates expression of MHC molecules may act, for instance, by increasing

-47-

transcription of MHC class I and/or class II genes, by increasing translation of MHC class I and/or class II mRNAs or by increasing stability or transport of MHC class I and/or class II proteins to the cell surface. A number of agents have been shown to increase the level of cell-surface expression of MHC class II molecules. See for example Cockfield, S.M. et al., *J. Immunol.* 144, 2967-2974 (1990); Noelle, R.J. et al. *J. Immunol.* 137, 1718-1723 (1986); Mond, J.J., et al., *J. Immunol.* 127, 881-888 (1981); Willman, C.L., et al. *J. Exp. Med.*, 170, 1559-1567 (1989); Celada, A. and Maki, R. *J. Immunol.* 146, 114-120 (1991) and Glimcher, L.H. and Kara, C.J. *Ann. Rev. Immunol.* 10, 13-49 (1992) and references therein. These agents include cytokines, antibodies to other cell surface molecules and phorbol esters. One agent which upregulates MHC class I and class II molecules on a wide variety of cell types is the cytokine interferon- γ . Thus, for example, tumor cells modified to express B7-2 and/or B7-3 and B7-1 can be further modified to increase expression of MHC molecules by contact with interferon- γ .

Another agent which can be used to induce or increase expression of an MHC molecule on a tumor cell surface is a nucleic acid encoding a transcription factor which upregulates transcription of MHC class I or class II genes. Such a nucleic acid can be transfected into the tumor cell to cause increased transcription of MHC genes, resulting in increased cell-surface levels of MHC proteins. MHC class I and class II genes are regulated by different transcription factors. However, the multiple MHC class I genes are regulated coordinately, as are the multiple MHC class II genes. Therefore, transfection of a tumor cell with a nucleic acid encoding a transcription factor which regulates MHC gene expression may increase expression of several different MHC molecules on the tumor cell surface. Several transcription factors which regulate the expression of MHC genes have been identified, cloned and characterized. For example, see Reith, W. et al., *Genes Dev.* 4, 1528-1540, (1990); Liou, H.-C., et al., *Science* 247, 1581-1584 (1988); Didier, D.K., et al., *Proc. Natl. Acad. Sci. USA* 85, 7322-7326 (1988).

(8). Inhibition of Invariant Chain Expression in Tumor Cells

Another embodiment of the invention provides a tumor cell modified to express a T cell costimulatory molecule (e.g., B7-2 and/or B7-3 and B7-1) and in which expression of an MHC class II-associated protein, the invariant chain, is inhibited. Invariant chain expression is inhibited to promote association of endogenously-derived TAA peptides with MHC class II molecules to create an antigen-MHC complex. This complex can trigger an antigen-specific signal in T cells to induce activation of T cells in conjunction with a costimulatory signal. MHC class II molecules have been shown to be capable of presenting endogenously-derived peptides. Nuchtern, J.G., et al. *Nature* 343, 74-76 (1990); Weiss, S. and Bogen, B. *Cell* 767-776 (1991). However, in cells which naturally express MHC class II molecules, the α and β

-48-

- chain proteins are associated with the invariant chain (hereafter Ii) during intracellular transport of the proteins from the endoplasmic reticulum. It is believed that Ii functions in part by preventing the association of endogenously-derived peptides with MHC class II molecules. Elliott, W., et al. *J. Immunol.* 138, 2949-2952 (1987); Stockinger, B., et al. *Cell* 56, 683-689 (1989); Guagliardi, L., et al. *Nature (London)* 343, 133-139 (1990); Bakke, O., et al. *Cell* 63, 707-716 (1990); Lottreau, V., et al. *Nature* 348, 600-605 (1990); Peters, J., et al. *Nature* 349, 669-676 (1991); Roche, P., et al. *Nature* 345, 615-618 (1990); Teyton, L., et al. *Nature* 348, 39-44 (1990). Since TAAs are synthesized endogenously in tumor cells, peptides derived from them are likely to be available intracellularly. Accordingly, inhibiting the expression of Ii in tumor cells which express Ii may increase the likelihood that TAA peptides will associate with MHC class II molecules. Consistent with this mechanism, it was shown that supertransfection of an MHC class II⁺, Ii⁻ tumor cell with the Ii gene prevented stimulation of tumor-specific immunity by the tumor cell. Clements, V.K., et al. *J. Immunol.* 149, 2391-2396 (1992).
- Prior to modification, the expression of Ii in a tumor cell can be assessed by detecting the presence or absence of Ii mRNA by Northern blotting or by detecting the presence or absence of Ii protein by immunoprecipitation. A preferred approach for inhibiting expression of Ii is by introducing into the tumor cells a nucleic acid which is antisense to a coding or regulatory region of the Ii gene, which have been previously described. Koch, N., et al., *EMBO J.* 6, 1677-1683, (1987). For example, an oligonucleotide complementary to nucleotides near the translation initiation site of the Ii mRNA can be synthesized. One or more antisense oligonucleotides can be added to media containing tumor cells, typically at a concentration of oligonucleotides of 200 µg/ml. The antisense oligonucleotide is taken up by tumor cells and hybridizes to Ii mRNA to prevent translation. In another embodiment, a recombinant expression vector is used in which a nucleic acid encoding sequences of the Ii gene in an orientation such that mRNA which is antisense to a coding or regulatory region of the Ii gene is produced. Tumor cells transfected with this recombinant expression vector thus contain a continuous source of Ii antisense nucleic acid to prevent production of Ii protein. Alternatively, Ii expression in a tumor cell can be inhibited by treating the tumor cell with an agent which interferes with Ii expression. For example, a pharmaceutical agent which inhibits Ii gene expression, Ii mRNA translation or Ii protein stability or intracellular transport can be used.

(9). Types of Tumor Cells to be Modified

- The tumor cells to be modified as described herein include tumor cells which can be transfected or treated by one or more of the approaches encompassed by the present invention to express B7-2 and/or B7-3, alone or in combination with B7-1. If necessary, the tumor

-49-

cells can be further modified to express MHC molecules or an inhibitor of Ii expression. A tumor from which tumor cells are obtained can be one that has arisen spontaneously, e.g. in a human subject, or may be experimentally derived or induced, e.g. in an animal subject. The tumor cells can be obtained, for example, from a solid tumor of an organ, such as a tumor of
5 the lung, liver, breast, colon, bone etc. Malignancies of solid organs include carcinomas, sarcomas, melanomas and neuroblastomas. The tumor cells can also be obtained from a blood-borne (i.e. dispersed) malignancy such as a lymphoma, a myeloma or a leukemia.

The tumor cells to be modified include those that express MHC molecules on their cell surface prior to transfection and those that express no or low levels of MHC class I
10 and/or class II molecules. A minority of normal cell types express MHC class II molecules. It is therefore expected that many tumor cells will not express MHC class II molecules naturally. These tumors can be modified to express B7-2 and/or B7-3 and MHC class II molecules. Several types of tumors have been found to naturally express surface MHC class II molecules, such as melanomas (van Duinen et al., *Cancer Res.* 48, 1019-1025, 1988),
15 diffuse large cell lymphomas (O'Keane et al., *Cancer* 66, 1147-1153, 1990), squamous cell carcinomas of the head and neck (Mattijsen et al., *Int. J. Cancer* 6, 95-100, 1991) and colorectal carcinomas (Moller et al., *Int. J. Cancer* 6, 155-162, 1991). Tumor cells which naturally express class II molecules can be modified to express B7-2 and/or B7-3, and, in addition, other class II molecules which can increase the spectrum of TAA peptides which
20 can be presented by the tumor cell. Most non-malignant cell types express MHC class I molecules. However, malignant transformation is often accompanied by downregulation of expression of MHC class I molecules on the surface of tumor cells. Csiba, A., et al., *Brit. J. Cancer* 50, 699-709 (1984). Importantly, loss of expression of MHC class I antigens by tumor cells is associated with a greater aggressiveness and/or metastatic potential of the
25 tumor cells. Schrier, P.I., et al. *Nature* 305, 771-775 (1983); Holden, C.A., et al. *J. Am. Acad. Dermatol.* 9., 867-871 (1983); Baniyash, M., et al. *J. Immunol.* 129, 1318-1323 (1982). Types of tumors in which MHC class I expression has been shown to be inhibited include melanomas, colorectal carcinomas and squamous cell carcinomas. van Duinen et al., *Cancer Res.* 48, 1019-1025, (1988); Moller et al., *Int. J. Cancer* 6, 155-162, (1991); Csiba, A., et al.,
30 *Brit. J. Cancer* 50, 699-709 (1984); Holden, C.A., et al. *J. Am. Acad. Dermatol.* 9., 867-871 (1983). A tumor cell which fails to express class I molecules or which expresses only low levels of MHC class I molecules can be modified by one or more of the techniques described herein to induce or increase expression of MHC class I molecules on the tumor cell surface to enhance tumor cell immunogenicity.

-50-

(10). Modification of Tumor Cells *In Vivo*

Another aspect of the invention provides methods for increasing the immunogenicity of a tumor cell by modification of the tumor cell *in vivo* to express B7-2 and/or B7-3 and B7-1 to trigger a costimulatory signal in T cells. In addition, tumor cells can be further modified 5 *in vivo* to express MHC molecules to trigger a primary, antigen-specific, signal in T cells. Tumor cells can be modified *in vivo* by introducing a nucleic acid encoding B7-2 and/or B7-3 and B7-1 into the tumor cells in a form suitable for expression of the costimulatory molecule(s) on the surface of the tumor cells. Likewise, nucleic acids encoding MHC class I or class II molecules or an antisense sequence of the *Ii* gene can be introduced into tumor 10 cells *in vivo*. In one embodiment, a recombinant expression vector is used to deliver nucleic acid encoding B7-2 and/or B7-3 and B7-1 to tumor cells *in vivo* as a form of gene therapy. Vectors useful for *in vivo* gene therapy have been previously described and include retroviral vectors, adenoviral vectors and adeno-associated viral vectors. See e.g. Rosenfeld, M.A., *Cell* 68, 143-155 (1992); Anderson, W.F., *Science* 226, 401-409 (1984); Friedman, T., 15 *Science* 244, 1275-1281 (1989). Alternatively, nucleic acid can be delivered to tumor cells *in vivo* by direct injection of naked nucleic acid into tumor cells. See e.g. Acsadi, G., et al., *Nature* 332, 815-818 (1991). A delivery apparatus is commercially available (BioRad). Optionally, to be suitable for injection, the nucleic acid can be complexed with a carrier such 20 as a liposome. Nucleic acid encoding an MHC class I molecule complexed with a liposome has been directly injected into tumors of melanoma patients. Hoffman, M., *Science* 256, 305-309 (1992).

Tumor cells can also be modified *in vivo* by use of an agent which induces or increases expression of B7-2 and/or B7-3 and B7-1 (and, if necessary, MHC molecules) as described herein. The agent may be administered systemically, e.g. by intravenous injection, 25 or, preferably, locally to the tumor cells.

(11). The Effector Phase of the Anti-Tumor T Cell-Mediated Immune Response

The modified tumor cells of the invention are useful for stimulating an anti-tumor T cell-mediated immune response by triggering an antigen-specific signal and a costimulatory 30 signal in tumor-specific T cells. Following this inductive, or afferent, phase of an immune response, effector populations of T cells are generated. These effector T cell populations can include both CD4+ T cells and CD8+ T cell. The effector populations are responsible for elimination of tumors cell, by, for example, cytosis of the tumor cells. Once T cells are activated, expression of a costimulatory molecule is not required on a target cell for 35 recognition of the target cell by effector T cells or for the effector functions of the T cells. Harding, F.A. and Allison, J.P. *J. Exp. Med.* 177, 1791-1796 (1993). Therefore, the anti-tumor T cell-mediated immune response induced by the modified tumor cells of the invention

-51-

is effective against both the modified tumor cells and unmodified tumor cells which do not express a costimulatory molecule.

- Additionally, the density and/or type of MHC molecules on the cell surface required for the afferent and efferent phases of a T cell-mediated immune response can differ. Fewer 5 MHC molecules, or only certain types of MHC molecules (e.g. MHC class I but not MHC class II) may be needed on a tumor cell for recognition by effector T cells than is needed for the initial activation of T cells. Therefore, tumor cells which naturally express low amounts of MHC molecules but are modified to express increased amounts of MHC molecules can induce a T cell-mediated immune response which is effective against the unmodified tumor 10 cells. Alternatively, tumor cells which naturally express MHC class I molecules but not MHC class II molecules which are then modified to express MHC class II molecules can induce a T cell-mediated immune response which includes effector T cell populations which can eliminate the parental MHC class I+, class II- tumor cells.

15 (12). Therapeutic Compositions of Tumor Cells

Another aspect of the invention is a composition of modified tumor cells in a biologically compatible form suitable for pharmaceutical administration to a subject *in vivo*. This composition comprises an amount of modified tumor cells and a physiologically acceptable carrier. The amount of modified tumor cells is selected to be therapeutically 20 effective. The term "biologically compatible form suitable for pharmaceutical administration *in vivo*" means that any toxic effects of the tumor cells are outweighed by the therapeutic effects of the tumor cells. A "physiologically acceptable carrier" is one which is biologically compatible with the subject. Examples of acceptable carriers include saline and aqueous buffer solutions. In all cases, the compositions must be sterile and must be fluid to the extent 25 that easy syringability exists. The term "subject" is intended to include living organisms in which tumors can arise or be experimentally induced. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof.

Administration of the therapeutic compositions of the present invention can be carried out using known procedures, at dosages and for periods of time effective to achieve the 30 desired result. For example, a therapeutically effective dose of modified tumor cells may vary according to such factors as age, sex and weight of the individual, the type of tumor cell and degree of tumor burden, and the immunological competency of the subject. Dosage regimens may be adjusted to provide optimum therapeutic responses. For instance, a single dose of modified tumor cells may be administered or several doses may be administered over 35 time. Administration may be by injection, including intravenous, intramuscular, intraperitoneal and subcutaneous injections.

-52-

(13). Activation of Tumor-specific T Lymphocytes *In Vitro*

Another approach to inducing or enhancing an anti-tumor T cell-mediated immune response by triggering a costimulatory signal in T cells is to obtain T lymphocytes from a tumor-bearing subject and activate them *in vitro* by stimulating them with tumor cells and a stimulatory form of B7-2 and/or B7-3, alone or in combination with B7-1. T cells can be obtained from a subject, for example, from peripheral blood. Peripheral blood can be further fractionated to remove red blood cells and enrich for or isolate T lymphocytes or T lymphocyte subpopulations. T cells can be activated *in vitro* by culturing the T cells with tumor cells obtained from the subject (e.g. from a biopsy or from peripheral blood in the case of blood-borne malignancies) together with a stimulatory form of B7-2 and/or B7-3 or, alternatively, by exposure to a modified tumor cell as described herein. The term "stimulatory form" means that the costimulatory molecule is capable of crosslinking its receptor on a T cell and triggering a costimulatory signal in T cells. The stimulatory form of the costimulatory molecule can be, for example, a soluble multivalent molecule or an immobilized form of the costimulatory molecule, for instance coupled to a solid support. Fragments, mutants or variants (e.g. fusion proteins) of B7-2 and/or B7-3 which retain the ability to trigger a costimulatory signal in T cells can also be used. In a preferred embodiment, a soluble extracellular portion of B7-2 and/or B7-3 is used to provide costimulation to the T cells. Following culturing of the T cells *in vitro* with tumor cells and B7-2 and/or B7-3, or a modified tumor cell, to activate tumor-specific T cells, the T cells can be administered to the subject, for example by intravenous injection.

(14). Therapeutic Uses of Modified Tumor Cells

The modified tumor cells of the present invention can be used to increase tumor immunogenicity, and therefore can be used therapeutically for inducing or enhancing T lymphocyte-mediated anti-tumor immunity in a subject with a tumor or at risk of developing a tumor. A method for treating a subject with a tumor involves obtaining tumor cells from the subject, modifying the tumor cells *ex vivo* to express a T cell costimulatory molecule, for example by transfecting them with an appropriate nucleic acid, and administering a therapeutically effective dose of the modified tumor cells to the subject. Appropriate nucleic acids to be introduced into a tumor cell include nucleic acids encoding B7-2 and/or B7-3, alone or together with nucleic acids encoding B7-1, MHC molecules (class I or class II) or Ii antisense sequences as described herein. Alternatively, after tumor cells are obtained from a subject, they can be modified *ex vivo* using an agent which induces or increases expression of B7-2 and/or B7-3 (and possibly also using agent(s) which induce or increase B7-1 or MHC molecules).

-53-

Tumor cells can be obtained from a subject by, for example, surgical removal of tumor cells, e.g. a biopsy of the tumor, or from a blood sample from the subject in cases of blood-borne malignancies. In the case of an experimentally induced tumor, the cells used to induce the tumor can be used, e.g. cells of a tumor cell line. Samples of solid tumors may be 5 treated prior to modification to produce a single-cell suspension of tumor cells for maximal efficiency of transfection. Possible treatments include manual dispersion of cells or enzymatic digestion of connective tissue fibers, e.g. by collagenase.

Tumor cells can be transfected immediately after being obtained from the subject or can be cultured *in vitro* prior to transfection to allow for further characterization of the tumor 10 cells (e.g. determination of the expression of cell surface molecules). The nucleic acids chosen for transfection can be determined following characterization of the proteins expressed by the tumor cell. For instance, expression of MHC proteins on the cell surface of the tumor cells and/or expression of the Ii protein in the tumor cell can be assessed. Tumors which express no, or limited amounts of or types of MHC molecules (class I or class II) can 15 be transfected with nucleic acids encoding MHC proteins; tumors which express Ii protein can be transfected with Ii antisense sequences. If necessary, following transfection, tumor cells can be screened for introduction of the nucleic acid by using a selectable marker (e.g. drug resistance) which is introduced into the tumor cells together with the nucleic acid of interest.

20 Prior to administration to the subject, the modified tumor cells can be treated to render them incapable of further proliferation in the subject, thereby preventing any possible outgrowth of the modified tumor cells. Possible treatments include irradiation or mitomycin C treatment, which abrogate the proliferative capacity of the tumor cells while maintaining the ability of the tumor cells to trigger antigen-specific and costimulatory signals in T cells 25 and thus to stimulate an immune response.

The modified tumor cells can be administered to the subject by injection of the tumor cells into the subject. The route of injection can be, for example, intravenous, intramuscular, intraperitoneal or subcutaneous. Administration of the modified tumor cells at the site of the original tumor may be beneficial for inducing local T cell-mediated immune responses 30 against the original tumor. Administration of the modified tumor cells in a disseminated manner, e.g. by intravenous injection, may provide systemic anti-tumor immunity and, furthermore, may protect against metastatic spread of tumor cells from the original site. The modified tumor cells can be administered to a subject prior to or in conjunction with other forms of therapy or can be administered after other treatments such as chemotherapy or 35 surgical intervention.

Additionally, more than one type of modified tumor cell can be administered to a subject. For example, an effective T cell response may require exposure of the T cell to more

-54-

- than one type of costimulatory molecule. Furthermore, the temporal sequence of exposure of the T cell to different costimulatory molecules may be important for generating an effective response. For example, it is known that upon activation, a B cell expresses B7-2 early in its response (about 24 hours after stimulation). Subsequently, B7-1 and B7-3 are expressed by 5 the B cell (about 48-72 hours after stimulation). Thus, a T cell may require exposure to B7-2 early in the induction of an immune response by exposure to B7-1 and/or B7-3 in the immune response. Accordingly, different types of modified tumor cells can be administered at different times to a subject to generate an effective immune response against the tumor cells. For example, tumor cells modified to express B7-2 can be administered to a subject.
- 10 Following this administration, a tumor cell from the same tumor but modified to express B7-3 (alone or in conjunction with B7-1) can be administered to the subject.

Another method for treating a subject with a tumor is to modify tumor cells *in vivo* to express B7-2 and/or B7-3, alone or in conjunction with B7-1, MHC molecules and/or an inhibitor of Ii expression. This method can involve modifying tumor cells *in vivo* by 15 providing nucleic acid encoding the protein(s) to be expressed using vectors and delivery methods effective for *in vivo* gene therapy as described in a previous section herein. Alternatively, one or more agents which induce or increase expression of B7-2 and/or B7-3, and possibly B7-1 or MHC molecules, can be administered to a subject with a tumor.

The modified tumor cells of the current invention may also be used in a method for 20 preventing or treating metastatic spread of a tumor or preventing or treating recurrence of a tumor. As demonstrated in detail in one of the following examples, anti-tumor immunity induced by B7-1-expressing tumor cells is effective against subsequent challenge by tumor cells, regardless of whether the tumor cells of the re-exposure express B7-1 or not. Thus, administration of modified tumor cells or modification of tumor cells *in vivo* as described 25 herein can provide tumor immunity against cells of the original, unmodified tumor as well as metastases of the original tumor or possible regrowth of the original tumor.

The current invention also provides a composition and a method for specifically inducing an anti-tumor response in CD4⁺ T cells. CD4⁺ T cells are activated by antigen in conjunction with MHC class II molecules. Association of peptidic fragments of TAAs with 30 MHC class II molecules results in recognition of these antigenic peptides by CD4⁺ T cells. Providing a subject with tumor cells which have been modified to express MHC class II molecules along with B7-2 and/or B7-3, or modified *in vivo* to express MHC class II molecules along with B7-2 and/or B7-3, can be useful for directing tumor antigen presentation to the MHC class II pathway and thereby result in antigen recognition by and 35 activation of CD4⁺ T cells specific for the tumor cells. Depletion of either CD4⁺ or CD8⁺ T cells *in vivo*, by administration of anti-CD4 or anti-CD8 antibodies, can be used to

-55-

demonstrate that specific anti-tumor immunity is mediated by a particular (e.g. CD4⁺) T cell subpopulation.

Subjects initially exposed to modified tumor cells develop an anti-tumor specific T cell response which is effective against subsequent exposure to unmodified tumor cells. Thus 5 the subject develops anti-tumor specific immunity. The generalized use of modified tumor cells of the invention from one human subject as an immunogen to induce anti-tumor immunity in another human subject is prohibited by histocompatibility differences between unrelated humans. However, use of modified tumor cells from one individual to induce anti-tumor immunity in another individual to protect against possible future occurrence of a tumor 10 may be useful in cases of familial malignancies. In this situation, the tumor-bearing donor of tumor cells to be modified is closely related to the (non-tumor bearing) recipient of the modified tumor cells and therefore the donor and recipient share MHC antigens. A strong hereditary component has been identified for certain types of malignancies, for example 15 certain breast and colon cancers. In families with a known susceptibility to a particular malignancy and in which one individual presently has a tumor, tumor cells from that individual could be modified to express B7-2 and/or B7-3, alone or in combination with B7-1 and administered to susceptible, histocompatible family members to induce an anti-tumor response in the recipient against the type of tumor to which the family is susceptible. This 20 anti-tumor response could provide protective immunity to subsequent development of a tumor in the immunized recipient.

(15). Tumor-Specific T Cell Tolerance

In the case of an experimentally induced tumor, a subject (e.g. a mouse) can be exposed to the modified tumor cells of the invention before being challenged with 25 unmodified tumor cells. Thus, the subject is initially exposed to TAA peptides on tumor cells together with B7-2 and/or B7-3, and B7-1 which activates TAA-specific T cells. The activated T cells are then effective against subsequent challenge with unmodified tumor cells. In the case of a spontaneously arising tumor, as is the case with human subjects, the subject's immune system will be exposed to unmodified tumor cells before exposure to the modified 30 tumor cells of the invention. Thus the subject is initially exposed to TAA peptides on tumor cells in the absence of a costimulatory signal. This situation is likely to induce TAA-specific T cell tolerance in those T cells which are exposed to and are in contact with the unmodified tumor cells. Secondary exposure of the subject to modified tumor cells which can trigger a costimulatory signal may not be sufficient to overcome tolerance in TAA-specific T cells 35 which were anergized by primary exposure to the tumor. Use of modified tumor cells to induce anti-tumor immunity in a subject already exposed to unmodified tumor cells may therefore be most effective in early diagnosed patients with small tumor burdens, for instance

-56-

- a small localized tumor which has not metastasized. In this situation, the tumor cells are confined to a limited area of the body and thus only a portion of the T cell repertoire may be exposed to tumor antigens and become anergized. Administration of modified tumor cells in a systemic manner, for instance after surgical removal of the localized tumor and
- 5 modification of isolated tumor cells, may expose non-anergized T cells to tumor antigens together with B7-2 and/or B7-3 alone, or in combination with B7-1 thereby inducing an anti-tumor response in the non-anergized T cells. The anti-tumor response may be effective against possible regrowth of the tumor or against micrometastases of the original tumor which may not have been detected. To overcome widespread peripheral T cell tolerance to
- 10 tumor cells in a subject, additional signals, such as a cytokine, may need to be provided to the subject together with the modified tumor cells. A cytokine which functions as a T cell growth factor, such as IL-2, could be provided to the subject together with the modified tumor cells. IL-2 has been shown to be capable of restoring the alloantigen-specific responses of previously anergized T cells in an *in vitro* system when exogenous IL-2 is added
- 15 at the time of secondary alloantigenic stimulation. Tan, P., et al. *J. Exp. Med.* 177, 165-173 (1993).

Another approach to generating an anti-tumor T cell response in a subject despite tolerance of the subject's T cells to the tumor is to stimulate an anti-tumor response in T cells from another subject who has not been exposed to the tumor (referred to as a naive donor) and transfer the stimulated T cells from the naive donor back into the tumor-bearing subject so that the transferred T cells can mount an immune response against the tumor cells. An anti-tumor response is induced in the T cells from the naive donor by stimulating the T cells *in vitro* with the modified tumor cells of the invention. Such an adoptive transfer approach is generally prohibited in outbred populations because of histocompatibility differences between the transferred T cells and the tumor-bearing recipient. However, advances in allogeneic bone marrow transplantation can be applied to this situation to allow for acceptance by the recipient of the adoptively transferred cells and prevention of graft versus host disease. First, a tumor-bearing subject (referred to as the host) is prepared for and receives an allogeneic bone marrow transplant from a naive donor by a known procedure. Preparation of the host involves whole body irradiation, which destroys the host's immune system, including T cells tolerized to the tumor, as well as the tumor cells themselves. Bone marrow transplantation is accompanied by treatment(s) to prevent graft versus host disease such as depletion of mature T cells from the bone marrow graft, treatment of the host with immunosuppressive drugs or treatment of the host with an agent, such as CTLA4Ig, to induce donor T cell tolerance to host tissues. Next, to provide anti-tumor specific T cells to the host which can respond against residual tumor cells in the host or regrowth or metastases of the original tumor in the host, T cells from the naive donor are stimulated *in vitro* with tumor cells from the host

-57-

- which have been modified, as described herein, to express B7-2 and/or B7-3. Thus, the donor T cells are initially exposed to tumor cells together with a costimulatory signal and therefore are activated to respond to the tumor cells. These activated anti-tumor specific T cells are then transferred to the host where they are reactive against unmodified tumor cells.
- 5 Since the host has been reconstituted with the donor's immune system, the host will not reject the transferred T cells and, additionally, the treatment of the host to prevent graft versus host disease will prevent reactivity of the transferred T cells with normal host tissues.

H. Administration of Therapeutic Forms of B Lymphocyte Antigens

10 The peptides of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to either enhance or suppress T cell mediated immune response. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the protein to be administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term subject is intended to include 15 living organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of a peptide having the activity of a novel B lymphocyte antigen as described herein can be in any pharmacological form including a therapeutically active amount of peptide alone or in combination with a peptide having the activity of another B lymphocyte 20 antigen and a pharmaceutically acceptable carrier. Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a peptide having B7-2 activity may vary according to factors such as the disease state, age, sex, and weight of the individual, and the 25 ability of peptide to elicit a desired response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active compound (e.g., peptide) may be administered in a convenient manner 30 such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

To administer a peptide having B7-2 activity by other than parenteral administration, 35 it may be necessary to coat the peptide with, or co-administer the peptide with, a material to prevent its inactivation. For example, a peptide having B7-2 activity may be administered to an individual in an appropriate carrier, diluent or adjuvant, co-administered with enzyme

-58-

inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasyloL. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) *J. Neuroimmunol* 7:27).

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound (e.g., peptide having B7-2 activity) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient (e.g., peptide) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

-59-

When the active compound is suitably protected, as described above, the protein may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents,
5 and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated.

Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form
10 for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly
15 dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

I. Identification of Cytokines Induced by Costimulation

20 The nucleic acid sequences encoding peptides having the activity of novel B lymphocyte antigens as described herein can be used to identify cytokines which are produced by T cells in response to stimulation by a form of B lymphocyte antigen, e.g., B7-2. T cells can be suboptimally stimulated *in vitro* with a primary activation signal, such as phorbol ester, anti-CD3 antibody or preferably antigen in association with an MHC class II molecule, and given a costimulatory signal by a stimulatory form of B7-2 antigen, for instance by a cell transfected with nucleic acid encoding a peptide having B7-2 activity and expressing the peptide on its surface or by a soluble, stimulatory form of the peptide. Known cytokines released into the media can be identified by ELISA or by the ability of an antibody which blocks the cytokine to inhibit T cell proliferation or proliferation of other cell types
25 that is induced by the cytokine. An IL-4 ELISA kit is available from Genzyme (Cambridge MA), as is an IL-7 blocking antibody. Blocking antibodies against IL-9 and IL-12 are available from Genetics Institute (Cambridge, MA).

An *in vitro* T cell costimulation assay as described above can also be used in a method for identifying novel cytokines which may be induced by costimulation. If a particular
35 activity induced upon costimulation, e.g., T cell proliferation, cannot be inhibited by addition of blocking antibodies to known cytokines, the activity may result from the action of an

-60-

unkown cytokine. Following costimulation, this cytokine could be purified from the media by conventional methods and its activity measured by its ability to induce T cell proliferation.

To identify cytokines which prevent the induction of tolerance, an *in vitro* T cell costimulation assay as described above can be used. In this case, T cells would be given the primary activation signal and contacted with a selected cytokine, but would not be given the costimulatory signal. After washing and resting the T cells, the cells would be rechallenged with both a primary activation signal and a costimulatory signal. If the T cells do not respond (e.g., proliferate or produce IL-2) they have become tolerized and the cytokine has not prevented the induction of tolerance. However, if the T cells respond, induction of tolerance has been prevented by the cytokine. Those cytokines which are capable of preventing the induction of tolerance can be targeted for blockage *in vivo* in conjunction with reagents which block B lymphocyte antigens as a more efficient means to induce tolerance in transplant recipients or subjects with autoimmune diseases. For example, one could administer a B7-2 blocking reagent together with a cytokine blocking antibody to a subject.

15

J. Identification of Molecules which Inhibit Costimulation

Another application of the peptide having the activity of a novel B lymphocyte antigen of the invention (e.g., B7-2 and B7-3) is the use of one or more of these peptides in screening assays to discover as yet undefined molecules which are inhibitors of costimulatory ligand binding and/or of intracellular signaling through T cells following costimulation. For example, a solid-phase binding assay using a peptide having the activity of a B lymphocyte antigen, such as B7-2, could be used to identify molecules which inhibit binding of the antigen with the appropriate T cell ligand (e.g., CTLA4, CD28). In addition, an *in vitro* T cell costimulation assay as described above could be used to identify molecules which interfere with intracellular signaling through the T cells following costimulation as determined by the ability of these molecules to inhibit T cell proliferation and/or cytokine production (yet which do not prevent binding of B lymphocyte antigens to their receptors). For example, the compound cyclosporine A inhibits T cell activation through stimulation via the T cell receptor pathway but not via the CD28/CTLA4 pathway. Therefore, a different intracellular signaling pathway is involved in costimulation. Molecules which interfere with intracellular signaling via the CD28/CTLA4 pathway may be effective as immunosuppressive agents *in vivo* (similar to the effects of cyclosporine A).

35

K. Identification of Molecules which Modulate B Lymphocyte Antigen Expression

The monoclonal antibodies produced using the proteins and peptides of the current invention can be used in a screening assay for molecules which modulate the expression of B lymphocyte antigens on cells. For example, molecules which effect intracellular signaling

-61-

- which leads to induction of B lymphocyte antigens, e.g. B7-2 or B7-3, can be identified by assaying expression of one or more B lymphocyte antigens on the cell surface. Reduced immunofluorescent staining by an anti-B7-2 antibody in the presence of the molecule would indicate that the molecule inhibits intracellular signals. Molecules which upregulate B
- 5 lymphocyte antigen expression result in an increased immunofluorescent staining.
- Alternatively, the effect of a molecule on expression of a B lymphocyte antigen, such as B7-2, can be determined by detecting cellular B7-2 mRNA levels using a B7-2 cDNA as a probe. For example, a cell which expresses a peptide having B7-2 activity can be contacted with a molecule to be tested, and an increase or decrease in B7-2 mRNA levels in the cell detected
- 10 by standard technique, such as Northern hybridization analysis or conventional dot blot of mRNA or total poly(A⁺)RNAs using a B7-2 cDNA probe labeled with a detectable marker. Molecules which modulate B lymphocyte antigen expression may be useful therapeutically for either upregulating or downregulating immune responses alone or in conjunction with soluble blocking or stimulating reagents. For instance, a molecule which inhibits expression
- 15 of B7-2 could be administered together with a B7-2 blocking reagent for immunosuppressive purposes. Molecules which can be tested in the above-described assays include cytokines such as IL-4, γ INF, IL-10, IL-12, GM-CSF and prostagladins.
- This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references and published patent applications cited
- 20 throughout this application are hereby incorporated by reference.

The following methodology was used in Examples 1, 2 and 3.

METHODS AND MATERIALS

A. Cells

Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation from single cell suspensions of normal human spleens and were separated into E- and E+ fractions by rosetting with sheep red blood cells (Boyd, A.W., et al. (1985) *J. Immunol.* 134, 1516). B cells were purified from the E- fraction by adherence of monocytes on plastic and depletion of residual T, natural killer cells (NK) and residual monocytes by two treatments with anti-MsIgG and anti-MsIgM coated magnetic beads (Advanced Magnetics, Cambridge, MA), using monoclonal antibodies: anti-CD4, -CD8, -CD11b, -CD14 and -CD16. CD4+ T cells were isolated from the E+ fraction of the same spleens after adherence on plastic and depletion of NK, B cells and residual monocytes with magnetic beads and monoclonal antibodies: anti-CD20, -CD11b, -CD8 and -CD16. CD28+ T cells were identically isolated from the E+ fraction using anti-CD20, -CD11b, -CD14 and -CD16 monoclonal antibodies. The efficiency of the purification was analyzed by indirect immunofluorescence and flow

-62-

cytometry using an EPICS flow cytometer (Coulter). B cell preparations were >95% CD20+, <2% CD3+, <1% CD14+. CD4+ T cell preparations were >98% CD3+, >98% CD4+, <1% CD8+, <1% CD20+, <1% CD14+. CD28+ T cell preparations were >98% CD3+, >98% CD28+, <1% CD20+, <1% CD14+.

5

B. Monoclonal Antibodies and Fusion Proteins

Monoclonal antibodies were used as purified Ig unless indicated otherwise: anti-B7:133, IgM is a blocking antibody and has been previously described (Freedman, A.S. et al. (1987) *Immunol.* 132, 3260-3267); anti-B7:B1.1, IgG1 (RepliGen Corp., Cambridge, MA) (Nickoloff, B., et al (1993) *Am. J. Pathol.* 142, 1029-1040) is a non-blocking monoclonal antibody; BB-1: IgM is a blocking antibody (Dr. E. Clark, University of Washington, Seattle, WA) (Yokochi, T., et al. (1982) *J. Immunol.* 128, 823-827); anti-CD20: B1, IgG2a (Stashenko, P., et al.(1980) *J. Immunol.* 125, 1678-1685); anti-B5: IgM (Freedman, A., et al. (1985) *J. Immunol.* 134, 2228-2235); anti-CD8: 7PT 3F9, IgG2a; anti-CD4: 19Thy5D7, IgG2a; anti-CD11b: Mo1, IgM and anti-CD14: Mo2, IgM (Todd, R, et al. (1981) *J. Immunol.* 126, 1435-1442); anti-MHC class II: 9-49, IgG2a (Dr R. Todd, University of Michigan, Ann Arbor) (Todd, R.I., et al. (1984) *Hum Immunol.* 10, 23-40; anti-CD28: 9.3, IgG2a (Dr. C. June, Naval Research Institute, Bethesda) (Hansen, J.A., et al. (1980) *Immunogenetics.* 10, 247-260); anti-CD16: 3G8, IgG1 (used as ascites) (Dr. J. Ritz, Dana-Farber Cancer Institute, Boston); anti-CD3: OKT3, IgG2a hybridoma was obtained from the American Type Culture Collection and the purified monoclonal antibody was adhered on plastic plates at a concentration of 1 μ g/ml; anti-CD28 Fab fragments were generated from the 9.3 monoclonal antibody, by papain digestion and purification on a protein A column, according to the manufacturer's instructions (Pierce, Rockford, IL). Human CTLA4 fusion protein (CTLA4Ig) and control fusion protein (control-Ig) were prepared as previously described (Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci USA* 90:6586-6590); Boussiotis, V., et al *J. Exp. Med.* (accepted for publication)).

C. CHO Cell Transfection

30 B7-1 transfectants (CHO-B7) were prepared from the B7-1 negative chinese hamster ovary (CHO) cell line, fixed with paraformaldehyde and used as previously described (Gimmi, C.D., et al. *Proc. Natl. Acad. Sci USA* 88, 6575-6579).

D. In Vitro B Cell Activation and Selection of B7+ and B7- Cells

35 Splenic B cells were cultured at 2x10⁶ cells/ml in complete culture media, {RPMI 1640 with 10% heat inactivated fetal calf serum (FCS), 2mM glutamine, 1 mM sodium pyruvate, penicillin (100 units/ml), streptomycin sulfate (100 μ g/ml) and gentamycin sulfate

-63-

(5 μ g/ml}), in tissue culture flasks and were activated by crosslinking of sIg with affinity purified rabbit anti-human IgM coupled to Affi-Gel 702 beads (Bio-Rad), Richmond, CA) (Boyd, A.W., et al., (1985) *J. Immunol.* 134,1516) or by crosslinking of MHC class II with 9-49 antibody coupled to Affi-Gel 702 beads. B cells activated for 72 hours, were used as total 5 activated B cell populations or were indirectly stained with anti-B7 (B1.1) monoclonal antibody and fluorescein isothiocyanate (FITC) labeled goat anti-mouse immunoglobulin (Fisher, Pittsburgh, PA), and fractionated into B7-1+ and B7-1- populations by flow cytometric cell sorting (EPICS Elite flow cytometer, Coulter).

10 **E. Immunofluorescence and Flow Cytometry**

For surface phenotype analysis populations of B cells activated by either sIg or MHC class II crosslinking for 6, 12, 24, 48, 72 and 96 hours were stained with either anti-B7 (133), BB-1 monoclonal antibodies, control IgM antibody, CTLA4Ig or control-Ig. Cell suspensions were stained by two step indirect membrane staining with 10 μ g/ml of primary 15 monoclonal antibody followed by the appropriate secondary reagents. Specifically, immunoreactivity with anti-B7 (133) and BB-1 monoclonal antibodies was studied by indirect staining using goat anti-mouse Ig or immunoglobulin FITC (Fisher) as secondary reagent and immunoreactivity with fusion proteins was studied using biotinylated CTLA4Ig or biotinylated control-Ig and streptavidin-phycerythrin as secondary reagent. PBS 20 containing 10% AB serum was used as diluent and wash media. Cells were fixed with 0.1% paraformaldehyde and analyzed on a flow cytometer (EPICS Elite Coulter).

F. Proliferation Assay

T cells were cultured at a concentration of 1x10⁵ cells per well in 96-well flat bottom 25 microtiter plate at 37°C for 3 days in 5% CO₂. Syngeneic activated B cells (total B cell population or B7+ and B7- fractions) were irradiated (2500 rad) and added into the cultures at a concentration of 1x10⁵ cells per well. Factors under study were added to the required concentration for a total final volume of 200 μ l per well. When indicated, T cells were 30 incubated with anti-CD28 Fab (final concentration of 10 μ g/ml), for 30 minutes at 4°C, prior to addition in experimental plates. Similarly, CHO-B7 or B cells were incubated with CTLA4Ig or control-Ig (10 μ g/ml) for 30 minutes at 4°C. Thymidine incorporation as an index of mitogenic activity, was assessed after incubation with 1 μ Ci (37kBq) of {methyl-³H} thymidine (Du Pont, Boston, MA) for the last 15 hours of the culture. The cells were 35 harvested onto filters and the radioactivity on the dried filters was measured in a Pharmacia beta plate liquid scintillation counter.

-64-

G. IL-2 and IL-4 Assay

IL-2 and IL-4 concentrations were assayed by ELISA (R&D Systems, Minneapolis, MN and BioSource, Camarillo, CA) in culture supernatants collected at 24 hours after initiation of the culture.

5

EXAMPLE 1

Expression of a Novel CTLA4 Ligand on Activated B Cells

Which Induces T Cell Proliferation

10

Since crosslinking surface Ig induces human resting B cells to express B7-1 maximally (50-80%) at 72 hours, the ability of activated human B lymphocytes to induce submitigenically activated T cells to proliferate and secrete IL-2 was determined. Figure 1 depicts the costimulatory response of human splenic CD28+ T cells, submitigenically activated with anti-CD3 monoclonal antibody, to either B7 (B7-1) transfected CHO cells (CHO-B7) or syngeneic splenic B cells activated with anti-Ig for 72 hours. 3 H-Thymidine incorporation was assessed for the last 15 hours of a 72 hours culture. IL-2 was assessed by ELISA in supernatants after 24 hours of culture (Detection limits of the assay: 31-2000 pg/ml). Figure 1 is representative of seventeen experiments.

15

Submitigenically activated CD28+ T cells proliferated and secreted high levels of IL-2 in response to B7-1 costimulation provided by CHO-B7 (Figure 1, panel a). Both

proliferation and IL-2 secretion were totally inhibited by blocking the B7-1 molecule on CHO cells with either anti-B7-1 monoclonal antibody or by a fusion protein for its high affinity receptor, CTLA4. Similarly, proliferation and IL-2 secretion were abrogated by

20 25 blocking B7-1 signalling via CD28 with Fab anti-CD28 monoclonal antibody. Control monoclonal antibody or control fusion protein had no effect. Nearly identical costimulation of proliferation and IL-2 secretion was provided by splenic B cells activated with anti-Ig for 72 hours (panel b). Though anti-B7-1 monoclonal antibody could completely abrogate both

proliferation and IL-2 secretion delivered by CHO-B7, anti-B7-1 monoclonal antibody consistently inhibited proliferation induced by activated B cells by only 50% whereas IL-2 secretion was totally inhibited. In contrast to the partial blockage of proliferation induced by

30 35 anti-B7-1 monoclonal antibody, both CTLA4Ig and Fab anti-CD28 monoclonal antibody completely blocked proliferation and IL-2 secretion. These results are consistent with the hypothesis that activated human B cells express one or more additional CTLA4/CD28

ligands which can induce T cell proliferation and IL-2 secretion.

-65-

EXAMPLE 2

Activated Human Splenic B Cells Express CTLA4 Ligand(s) Distinct from B7-1

In light of the above observations, whether other CTLA4 binding counter-receptors were expressed on activated B cells was determined. To this end, human splenic B cells were activated for 72 hours with anti-Ig and then stained with an anti-B7-1 monoclonal antibody (B1.1) which does not inhibit B7-1 mediated costimulation. Fluorescein isothiocyanate (FITC) and mAb B1.1 were used with flow cytometric cell sorting to isolate B7-1⁺ and B7-1⁻ fractions. The resulting post-sort positive population was 99% B7-1⁺ and the post-sort negative population was 98% B7-1⁻ (Figure 2).

To examine the costimulatory potential of each population, human splenic CD28⁺ T cells were submitogenically stimulated with anti-CD3 monoclonal antibody in the presence of irradiated B7-1+ or B7-1- anti-Ig activated (72 hours) splenic B cells. ³H-Thymidine incorporation was assessed for the last 15 hours of a 72 hours culture. IL-2 was assessed by ELISA in supernatants after 24 hours of culture (Detection limits of the assay: 31-2000 pg/ml). The results of Figure 3 are representative of ten experiments. B7-1+ B cells induced anti-CD3 activated T cells to proliferate and secrete IL-2 (Figure 3a) but not IL-4. As was observed with the unfractionated activated B cell population, anti-B7-1 monoclonal antibody (133) inhibited proliferation only 50% but consistently abrogated IL-2 secretion. As above, CTLA4Ig binding or blockade of CD28 with Fab anti-CD28 monoclonal antibody completely inhibited both proliferation and IL-2 secretion. Control monoclonal antibody and control-Ig were not inhibitory. In an attempt to identify other potential CTLA4/CD28 binding costimulatory ligand(s) which might account for the residual, non-B7 mediated proliferation delivered by B7+ B cells, the effect of BB-1 monoclonal antibody on proliferation and IL-2 secretion was examined. As seen, BB-1 monoclonal antibody completely inhibited both proliferation and IL-2 secretion (Figure 3a). Figure 3b displays the costimulatory potential of B7-1- activated human splenic B cells. Irradiated B7-1- activated (72 hr) B cells could also deliver a significant costimulatory signal to submitogenically activated CD4⁺ lymphocytes. This costimulation was not accompanied by detectable IL-2 (Figure 3b) or IL-4 accumulation and anti-B7-1 monoclonal antibody did not inhibit proliferation. However, CTLA4Ig, Fab anti-CD28 monoclonal antibody, and BB-1 monoclonal antibody all completely inhibited proliferation.

Phenotypic analysis of the B7-1+ and B7-1- activated splenic B cells confirmed the above functional results. Figure 4 shows the cell surface expression of B7-1, B7-2 and B7-3 on fractionated B7-1⁺ and B7-1⁻ activated B cell. As seen in Figure 4, B7-1+ activated splenic B cells stained with anti-B7-1 (133) monoclonal antibody, BB-1 monoclonal antibody, and bound CTLA4-Ig. In contrast, B7- activated splenic B cells did not stain with

-66-

anti-B7-1 (133) monoclonal antibody but did stain with BB-1 monoclonal antibody and CTLA4Ig. These phenotypic and functional results demonstrate that both B7-1+ and B7-1- activated (72 hours) human B lymphocytes express CTLA4 binding counter-receptor(s) which: 1) can induce submitogenically activated T cells to proliferate without detectable IL-5 secretion; and 2) are identified by the BB-1 monoclonal antibody but not anti-B7-1 monoclonal antibody. Thus, these CTLA4/CD28 ligands can be distinguished on the basis of their temporal expression after B cell activation and their reactivity with CTLA4Ig and anti-B7 monoclonal antibodies. The results of Figure 4 are representative of five experiments.

10

EXAMPLE 3

Three Distinct CTLA4/CD28 Ligands Are Expressed Following Human B Cell Activation

To determine the sequential expression of CTLA4 binding counter-receptors following activation, human splenic B cells were activated by crosslinking of either surface Ig or MHC class II and the expression of B7-1, B7-3 and B7-2 binding proteins were examined by flow cytometric analysis. Ig or MHC class II crosslinking induced a similar pattern of CTLA4Ig binding (Figures 5 and 6). Figure 5 is representative of the results of 25 experiments for anti-B7-1 and BB-1 binding and 5 experiments for CTLA4Ig binding. Figure 6 is representative of 25 experiments for anti-B7-1 binding and 5 experiments for CTLA4Ig binding. The results of these experiments indicate that prior to 24 hours, none of these molecules are expressed. At 24 hours post-activation, the majority of cells express a protein that binds CTLA4Ig (B7-2), however, fewer than 20% express either B7-1 or B7-3. Crosslinking of MHC class II induces maximal expression and intensity of B7-1 and B7-3 at 48 hours whereas crosslinking of Ig induces maximal expression at 72 hours and expression declines thereafter. These results suggest that an additional CTLA4 binding counter-receptor is expressed by 24 hours and that the temporal expression of the distinct B7-1 and B7-3 proteins appears to coincide.

A series of experiments was conducted to determine whether the temporal expression of CTLA4 binding counter-receptors differentially correlated with their ability to costimulate T cell proliferation and/or IL-2 secretion. Human splenic CD28+ T cells submitogenically stimulated with anti-CD3 were cultured for 72 hours in the presence of irradiated human splenic B cells that had been previously activated *in vitro* by sIg crosslinking for 24, 48, or 72 hours. IL-2 secretion was assessed by ELISA in supernatants after 24 hours and T cell proliferation as assessed by ³H-thymidine incorporation for the last 15 hours of a 72 hour culture. The results of Figure 7 are representative of 5 experiments. As seen in Figure 7a, 24 hour activated B cells provided a costimulatory signal which was accompanied by modest

-67-

levels of IL-2 production, although the magnitude of proliferation was significantly less than observed with 48 and 72 hours activated human B cells (note differences in scale for ³H-Thymidine incorporation). Neither proliferation nor IL-2 accumulation was inhibited by anti-B7-1 (133) or BB-1. In contrast, with CTLA4Ig and anti-CD28 Fab monoclonal antibody totally abrogated proliferation and IL-2 accumulation. B cells activated for 48 hours, provided costimulation which resulted in nearly maximal proliferation and IL-2 secretion (Figure 7b). Here, anti-B7-1 (133) monoclonal antibody, inhibited proliferation approximately 50% but totally blocked IL-2 accumulation. BB-1 monoclonal antibody totally inhibited both proliferation and IL-2 secretion. As above, CTLA4Ig and Fab 5 antibody also totally blocked proliferation and IL-2 production. Finally, 72 hour activated B cells induced T cell response identical to that induced by 48 hour activated B cells. Similar results are observed if the submitogenic signal is delivered by phorbol myristic acid (PMA) and if the human splenic B cells are activated by MHC class II rather than Ig crosslinking. These results indicate that there are three CTLA4 binding molecules that are temporarily 10 expressed on activated B cells and each can induce submitogenically stimulated T cells to proliferate. Two of these molecules, the early CTLA4 binding counter-receptor (B7-2) and B7-1 (133) induce IL-2 production whereas B7-3 induces proliferation without detectable 15 IL-2 production.

Previous studies provided conflicting evidence whether the anti-B7 monoclonal antibody, 133 and monoclonal antibody BB-1 identified the same molecule (Freedman, A.S. et al. (1987) *Immunol.* 137, 3260-3267; Yokochi, T., et al. (1982) *J. Immunol.* 128, 823-827; Freeman, G.J., et al. (1989) *J. Immunol.* 143, 2714-2722.). Although both monoclonal antibodies identified molecules expressed 48 hours following human B-cell activation, several reports suggested that B7 (B7-1) and the molecule identified by monoclonal antibody 20 BB-1 were distinct since they were differentially expressed on cell lines and B cell neoplasms (Freedman, A.S. et al. (1987) *Immunol.* 137, 3260-3267; Yokochi, T., et al. (1982) *J. Immunol.* 128, 823-827; Freeman, G.J., et al. (1989) *J. Immunol.* 143, 2714-2722; Clark, E and Yokochi, T. (1984) *Leukocyte Typing, 1st International References Workshop.* 339-346; Clark, E., et al. (1984) *Leukocyte Typing, 1st International References Workshop.* 740). In addition, immunoprecipitation and Western Blotting with these IgM monoclonal antibodies 25 suggested that they identified different molecules (Clark, E and Yokochi, T. (1984) *Leukocyte Typing, 1st International References Workshop.* 339-346; Clark, E., et al. (1984) *Leukocyte Typing, 1st International References Workshop.* 740). The original anti-B7 monoclonal antibody, 133, was generated by immunization with anti-immunoglobulin 30 activated human B lymphocytes whereas the BB-1 monoclonal antibody was generated by immunization with a baboon cell line. Thus, the BB-1 monoclonal antibody must identify an epitope on human cells that is conserved between baboons and humans.

-68-

Following the molecular cloning and expression of the human B7 gene (B7-1), B7 transfected COS cells were found to be identically stained with the anti-B7 (133) and BB-1 monoclonal antibodies and that they both precipitated the identical broad molecular band (44-54kD) strongly suggesting that they identified the same molecule (Freeman, G.J., et al. 5 (1989) *J. Immunol.* **143**, 2714-2722). This observation was unexpected since the gene encoding the molecule identified by the BB-1 monoclonal antibody had been previously mapped to chromosome 12 (Katz, F.E., et al. (1985) *Eur. J. Immunol.* **10** 3-6), whereas the B7 gene was located by two groups on chromosome 3 (Freeman, G.J., et al. (1992) *Blood* **79**, 489-494; Selvakumar, A., et al. (1992) *Immunogenetics* **36**, 175-181.). Subsequently, 10 additional discrepancies between the phenotypic expression of B7 (B7-1) and the molecule identified by the BB-1 monoclonal antibody were noted. BB-1 monoclonal antibody stained thymic epithelial cells (Turka, L.A., et al. (1991) *J. Immunol.* **146**, 1428-36; Munro, J.M., et al. *Blood* submitted.) and keratinocytes (Nickoloff, B., et al (1993) *Am. J. Pathol.* **142**, 1029-1040; Augustin, M., et al. (1993) *J. Invest. Dermatol.* **100**, 275-281.) whereas anti-B7 did 15 not. Recently, Nickoloff et al. (1993) *Am. J. Pathol.* **142**, 1029-1040, reported discordant expression of the molecule identified by the BB-1 monoclonal antibody and B7 on keratinocytes using a BB-1 and anti-B7 (B1.1 and 133) monoclonal antibodies. Nickoloff et al. also demonstrated that these BB-1 positive cells did not express B7 mRNA yet bound CD28 transfected COS cells providing further support for the existence of a distinct protein 20 which binds monoclonal antibody BB-1.

The present findings confirm that there is an additional CTLA4 counter-receptor identified by the BB-1 monoclonal antibody, B7-3, and that this protein appears to be functionally distinct from B7-1 (133). Although the expression of B7-1 and B7-3 following B cell activation appears to be concordant on B7 positive B cells, these studies demonstrate 25 that the B7-3 molecule is also expressed on B7 negative activated B cells. More importantly, the B7-3 molecule appears to be capable of inducing T cell proliferation without detectable IL-2 or IL-4 production. This result is similar to the previous observation that ICAM-1 could costimulate T cell proliferation without detectable IL-2 or IL-4 production (Boussiotis, V., et al *J. Exp. Med.* (accepted for publication)). These data indicate that the BB-1 monoclonal 30 antibody recognizes an epitope on the B7-1 protein and that this epitope is also found on a distinct B7-3 protein, which also has costimulatory function. Phenotypic and blocking studies demonstrate that the BB-1 monoclonal antibody could detect one (on B7 negative cells) or both (on B7 positive cells) of these proteins. In contrast, the anti-B7 monoclonal antibodies, 133 and B1.1 detect only the B7-1 protein. Taken together, these results suggest 35 that by 48 hours post B-cell activation by crosslinking of surface immunoglobulin or MHC class II, B cells express at least two distinct CTLA4 binding counter-receptors, one identified

-69-

by both anti-B7 and BB-1 monoclonal antibodies and the other identified only by BB-1 monoclonal antibody.

The B7-2 antigen is not detectable on activated B cells after 12 hours, but by 24 hours it is strongly expressed and functional. This molecule appears to signal via CD28 since 5 proliferation and IL-2 production are completely blocked by Fab anti-CD28 monoclonal antibody. At 48 hours post activation, IL-2 secretion seems to be accounted for by B7-1 costimulation, since anti-B7 monoclonal antibody completely inhibits IL-2 production.

Previous studies and results presented here demonstrate that B7 (B7-1) is neither expressed (Freedman, A.S. et al. (1987) *Immunol.* **137**, 3260-3267; Freedman, A.S., et al. 10 (1991) *Cell. Immunol.* **137**, 429-437) nor capable of costimulating T cell proliferation or IL-2 secretion until 48 hours post B-cell activation. Previous studies have shown that activation of T cells via the TCR in the absence of costimulation (Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci USA* **90**:6586-6590; Schwartz, R.H., et al. (1989) *Cold Spring Harb. Symp. Quant. Biol* **54**, 605-10; Beverly, B., et al. (1992) *Int. Immunol.* **4**, 661-671.) and lack of IL-2 15 (Boussiotis, V., et al *J. Exp. Med.* (submitted); Beverly, B., et al. (1992) *Int. Immunol.* **4**, 661-671; Wood, M., et al. (1993) *J. Exp. Med.* **177**, 597-603) results in anergy. If B7-1 were the only costimulatory molecule capable of inducing IL-2 secretion, T cells would be anergized within the first 24 hours following activation since there is no B7-1 present to costimulate IL-2 production. Therefore, the existence of another, early inducible costimulatory molecule, 20 which can costimulate IL-2 secretion during the first 24 hours would be necessary to induce an effective immune response rather than anergy. The appearance of the early CTLA4 binding counter-receptor, B7-2, between 12 and 24 hours post B cell activation, fulfills this function.

Two observations shed light on the biologic and potential clinical significance of 25 these two additional CTLA4 binding counter-receptors. First, B7 (B7-1) deficient mouse has been developed and its antigen presenting cells were found to still bind CTLA4Ig (Freeman and Sharpe manuscript in preparation). This mouse is viable and isolated mononuclear cells induce detectable levels of IL-2 when cultured with T cells *in vitro*. Therefore, an alternative CD28 costimulatory counter-receptor or an alternative IL-2 producing pathway must be 30 functional. Second, thus far the most effective reagents to induce antigen specific anergy in murine and human systems are CTLA4Ig and Fab anti-CD28, whereas anti-B7 monoclonal antibodies have been much less effective (Harding, F.A., et al. (1992) *Nature.* **356**, 607-609; Lenschow, D.J., et al. (1992) *Science.* **257**, 789-792; Chen, L., et al. (1992) *Cell.* **71**, 1093-1102; Tan, P., et al. (1993) *J. Exp. Med.* **177**, 165-173.). These observations are also 35 consistent with the hypothesis that alternative CTLA4/CD28 ligands capable of inducing IL-2 exist, and taken together with the results presented herein, suggest that all three CTLA4 binding counter-receptors may be critical for the induction of T cell immunity. Furthermore,

-70-

their blockade will likely be required for the induction of T cell anergy. Identical results have been observed in the murine system with the identification of two CTLA4 binding ligands, corresponding to the human B7-1 and B7-2 molecules. APCs in the B7 deficient mouse bind to the CTLA4 and can induce IL-2 secretion. Taken together, these observations 5 indicate that multiple CTLA-4 binding counter-receptors exist and sequentially costimulate T cell activation in the murine system.

EXAMPLE 4

Cloning, Sequencing and Expression of the B7-2 Antigen

10

A. Construction of cDNA Library

A cDNA library was constructed in the pCDM8 vector (Seed, *Nature*, 329:840 (1987)) using poly (A)⁺ RNA from the human anti-IgM activated B cells as described (Aruffo et al, *Proc. Natl. Acad. Sci. USA*, 84:3365 (1987)). Splenic B cells were cultured at 15 2x10⁶ cells/ml in complete culture media, (RPMI 1640 with 10% heat inactivated fetal calf serum (FCS), 2mM glutamine, 1 mM sodium pyruvate, penicillin (100 units/ml), streptomycin sulfate (100μg/ml) and gentamycin sulfate (5μg/ml)), in tissue culture flasks and were activated by crosslinking of slg with affinity purified rabbit anti-human IgM coupled to Affi-Gel 702 beads (Bio-Rad), Richmond, CA) (Boyd, A.W., et al., (1985) *J. Immunol.* 134,1516). Activated B cells were harvested after 1/6, 1/2, 4, 8 12, 24, 48, 72 and 96 hours.

RNA was prepared by homogenizing activated B cells in a solution of 4M guanidine thiocyanate, 0.5% sarkosyl, 25mM EDTA, pH 7.5, 0.13% Sigma anti-foam A, and 0.7% mercaptoethanol. RNA was purified from the homogenate by centrifugation for 24 hour at 25 32,000 rpm through a solution of 5.7M CsCl, 10mM EDTA, 25mM Na acetate, pH 7. The pellet of RNA was dissolved in 5% sarkosyl, 1mM EDTA, 10mM Tris, pH 7.5 and extracted with two volumes of 50% phenol, 49% chloroform, 1% isoamyl alcohol. RNA was ethanol precipitated twice. Poly (A)⁺ RNA used in cDNA library construction was purified by two cycles of oligo (dT)-cellulose selection.

30 Complementary DNA was synthesized from 5.5μg of anti-IgM activated human B cell poly(A)⁺ RNA in a reaction containing 50mM Tris, pH 8.3, 75mM KCl, 3mM MgCl₂, 10mM dithiothreitol, 500μM dATP, dCTP, dGTP, dTTP, 50μg/ml oligo(dT)12-18, 180 units/ml RNasin, and 10,000 units/ml Moloney-MLV reverse transcriptase in a total volume of 55μl at 37° for 1 hr. Following reverse transcription, the cDNA was converted to double-stranded DNA by adjusting the solution to 25mM Tris, pH 8.3, 100mM KCl, 5mM MgCl₂, 250μM each dATP, dCTP, dGTP, dTTP, 5mM dithiothreitol, 250 units/ml DNA polymerase I, 8.5 units/ml ribonuclease H and incubating at 16° for 2 hr. EDTA was added to 18mM and 35

-71-

the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate and with 4 micrograms of linear polyacrylamide as carrier. In addition, cDNA was synthesized from 4 μ g of anti-IgM activated human B cell poly(A)⁺ RNA in a reaction containing 50mM Tris, pH 8.8, 50 μ g/ml oligo(dT)12-18, 327 units/ml RNasin, and 952 units/ml AMV reverse transcriptase in a total volume of 100 μ l at 42° for 0.67 hr.

Following reverse transcription, the reverse transcriptase was inactivated by heating at 70° for 10 min. The cDNA was converted to double-stranded DNA by adding 320 μ l H₂O and 80 μ l of a solution of 0.1M Tris, pH 7.5, 25mM MgCl₂, 0.5M KCl, 250 μ g/ml bovine serum albumin, and 50mM dithiothreitol, and adjusting the solution to 200 μ M each dATP, dCTP, dGTP, dTTP, 50 units/ml DNA polymerase I, 8 units/ml ribonuclease H and incubating at 16° C for 2 hours. EDTA was added to 18 mM and the solution was extracted with an equal volume of 50 % phenol, 49 % chloroform, 1 % isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate and with 4 micrograms of linear polyacrylamide as carrier.

The DNA from 4 μ g of AMV reverse transcription and 2 μ g of Moloney MLV reverse transcription was combined. Non-selfcomplementary BstXI adaptors were added to the DNA as follows: The double-stranded cDNA from 6 μ g of poly(A)⁺ RNA was incubated with 3.6 μ g of a kinased oligonucleotide of the sequence CTTTAGAGCACA (SEQ ID NO:15) and 2.4 μ g of a kinased oligonucleotide of the sequence CTCTAAAG (SEQ ID NO:16) in a solution containing 6mM Tris, pH 7.5, 6mM MgCl₂, 5mM NaCl, 350 μ g/ml bovine serum albumin, 7mM mercaptoethanol, 0.1mM ATP, 2mM dithiothreitol, 1mM spermidine, and 600 units T4 DNA ligase in a total volume of 0.45ml at 15° C for 16 hours. EDTA was added to 34mM and the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate.

DNA larger than 600bp was selected as follows: The adaptored DNA was redissolved in 10mM Tris, pH 8, 1mM EDTA, 600mM NaCl, 0.1% sarkosyl and chromatographed on a Sepharose CL-4B column in the same buffer. DNA in the void volume of the column (containing DNA greater than 600bp) was pooled and ethanol precipitated.

The pCDM8 vector was prepared for cDNA cloning by digestion with BstXI and purification on an agarose gel. Adaptored DNA from 6 μ g of poly(A)⁺RNA was ligated to 2.25 μ g of BstXI cut pCDM8 in a solution containing 6mM Tris, pH 7.5, 6mM MgCl₂, 5mM NaCl, 350 μ g/ml bovine serum albumin, 7mM mercaptoethanol, 0.1mM ATP, 2mM dithiothreitol, 1mM spermidine, and 600 units T4 DNA ligase in a total volume of 1.5ml at 15° for 24 hr. The ligation reaction mixture was transformed into competent E.coli MC1061/P3 and a total of 4,290,000 independent cDNA clones were obtained.

-72-

Plasmid DNA was prepared from a 500 ml culture of the original transformation of the cDNA library. Plasmid DNA was purified by the alkaline lysis procedure followed by twice banding in CsCl equilibrium gradients (Maniatis et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1987)).

5

B. Cloning Procedure

In the first round of screening, thirty 100 mm dishes of 50% confluent COS cells were transfected with 0.05 μ g/ml anti-IgM activated human B cells library DNA using the DEAE-Dextran method (Seed et al, *Proc. Natl. Acad. Sci. USA*, 84:3365 (1987)). The cells were 10 trypsinized and re-plated after 24 hours. After 47 hours, the cells were detached by incubation in PBS/0.5 mM EDTA, pH 7.4/0.02% Na azide at 37°C for 30 min. The detached cells were treated with 10 μ g/ml/CTLA4Ig and CD28Ig for 45 minutes at 4°C. Cells were washed and distributed into panning dishes coated with affinity-purified Goat anti-human IgG antibody and allowed to attach at room temperature. After 3 hours, the plates were 15 gently washed twice with PBS/0.5mM EDTA, pH 7.4/0.02% Na azide, 5% FCS and once with 0.15M NaCl, 0.01 M Hepes, pH 7.4, 5% FCS. Episomal DNA was recovered from the panned cells and transformed into *E. coli* DH10B/P3. The plasmid DNA was re-introduced into COS cells via spheroplast fusion as described (Seed et al, *Proc. Natl. Acad. Sci. USA*, 84:3365 (1987)) and the cycle of expression and panning was repeated twice. In the second 20 and third rounds of selection, after 47 hours, the detached COS cells were first incubated with α -B7-1 mAbs (133 and B1.1, 10 μ g/ml), and COS cells expressing B7-1 were removed by α -mouse IgG and IgM coated magnetic beads. COS cells were then treated with 10 μ g/ml of human CTLA4Ig (hCTLA4Ig) and human CD28Ig (hCD28Ig) and human B7-2 expressing COS cells were selected by panning on dishes with goat anti-human IgG antibody plates. 25 After the third round, plasmid DNA was prepared from individual colonies and transfected into COS cells by the DEAE-Dextran method. Expression of B7-2 on transfected COS cells was analyzed by indirect immunofluorescence with CTLA4Ig.

After the final round of selection, plasmid DNA was prepared from individual colonies. A total of 4 of 48 candidate clones contained a cDNA insert of approximately 1.2 30 kb. Plasmid DNA from these four clones was transfected into COS cells. All four clones were strongly positive for B7-2 expression by indirect immunofluorescence using CTLA4Ig and flow cytometric analysis.

C. Sequencing

35 The B7-2 cDNA insert in clone29 was sequenced in the pCDM8 expression vector employing the following strategy. Initial sequencing was performed using sequencing primers T7, CDM8R (Invitrogen) homologous to pCDM8 vector sequences adjacent to the

-73-

cloned B7-2 cDNA (see Table I). Sequencing was performed using dye terminator chemistry and an ABI automated DNA sequencer. (ABI, Foster City, CA). DNA sequence obtained using these primers was used to design additional sequencing primers (see Table I). This cycle of sequencing and selection of additional primers was continued until the B7-2 cDNA
 5 was completely sequenced on both strands.

TABLE I

	T7(F) (SEQ ID NO:3)	5'd[TAATACGACTCACTATAGGG]3'
10	CDM8(R) (SEQ ID NO:4)	5'd[TAAAGGTTCCCTCACAAAG]3'
	CDM8 RGV(2) (SEQ ID NO:5)	5'd[ACTGGTAGGTATGGAAGATCC]3'
	HBX29-5P (2R) (SEQ ID NO:6)	5'd[ATGCGAATCATTCTGTGGGC]3'
	HBX29-5P (2F) (SEQ ID NO:7)	5'd[AAAGCCCACAGGAATGATTG]3'
	HBX29-5P (SEQ ID NO:8)	5'd[CTCTCAAAACCAAABCCTGAG]3'
15	SPA (SEQ ID NO:9)	5'd[TTAGGTCACAGCAGAACGAGC]3'
	SPA (3FA) (SEQ ID NO:10)	5'd[TCTGGAAACTGACAAGACGCG]3'
	HBX29-5P(1R) (SEQ ID NO:11)	5'd[CTCAGGCTTGGTTTGAGAG]3'
	HBX29-3P(1R) (SEQ ID NO:12)	5'd[CACTCTTCCCTCTCCATTG]3'
	HBX29-5P(3R) (SEQ ID NO:13)	5'd[GACAAGCTGATGGAAACGTCG]3'
20	HBX29-3P(1P) (SEQ ID NO:14)	5'd[CAATGGAGAGGGAAAGAGAGTG]3'

The human B7-2 clone 29 contained an insert of 1,120 base pairs with a single long open reading frame of 987 nucleotides and approximately 27 nucleotides of 3' noncoding sequences (Figure 8 (SEQ ID NO:1)). The predicted amino acid sequence encoded by the open reading frame of the protein is shown below the nucleotide sequence in Figure 8. The encoded protein, human B7-2, is predicted to be 329 amino acids in length (SEQ ID NO:2). This protein sequence exhibits many features common to other type 1 Ig superfamily membrane proteins. Protein translation is predicted to begin at the ATG codon (nucleotide
 25 107-109) based on DNA homology in this region with the consensus eukaryotic translation initiation site (Kozak, M. (1987) *Nucl. Acids Res.* **15**:8125-8148). The amino terminus of the human B7-2 protein (amino acids 1 to 23) has the characteristics of a secretory signal peptide with a predicted cleavage between the alanines at positions 23 and 24 (von Heijne (1986) *Nucl. Acids Res.* **14**:4683). Processing at this site would result in a human B7-2 membrane
 30 bound protein of 306 amino acid with an unmodified molecular weight of approximately 34 kDa. This protein would consist of an extracellular Ig superfamily V and C like domains, of from about amino acid residue 24-245, a hydrophobic transmembrane domain of from about
 35

-74-

amino acid residue 246-268 and a long cytoplasmic domain of from about amino acid residue 269-329. The homologies to the Ig superfamily are due to the two contiguous Ig-like domains in the extracellular region bound by the cysteines at positions 40 to 110 and 157 to 218. The extracellular domain also contains eight potential N-linked glycosylation sites. *E. coli* transfected with a vector containing the cDNA insert of clone 29, encoding the human B7-2 protein, was deposited with the American Type Culture Collection (ATCC) on July 26, 1993 as Accession No. 69357.

Comparison of both the nucleotide and amino acid sequences of human B7-2 with the GenBank and EMBL databases showed that only the human and murine B7-1 proteins are related. Alignment of the three B7 protein sequences (see Figure 13) shows that human B7-2 has approximately 26% amino acid identity with human B7-1. Figure 13 represents the comparison of the amino acid sequences for human B7-2 (hB7-2) (SEQ ID NO:2), human B7-1 (hB7-1) (SEQ ID NO: 28 and 29) and murine B7 (mB7) (SEQ ID NO: 30 and 31). The amino acid sequences for the human B7-1 and murine B7 (referred to herein as murine B7-1) can be found in Genbank at Accession #M27533 and X60958 respectively. Vertical lines in Figure 13 show identical amino acids between the hB7-2 and hB7-1 or mB7. Identical amino acids between hB7-1 and mB7 are not shown. The hB7-2 protein exhibits the same general structure as hB7-1 as defined by the common cysteines (positions 40 and 110, IgV domains; positions 157 and 217, IgC domain) which the Ig superfamily domains and by many other common amino acids. Since both hB7-1 and mB7 have been shown to bind to both human CTLA4 and human CD28, the amino acids in common between these two related proteins will be those necessary to comprise a CTLA4 or CD28 binding sequence. An example of such a sequence would be the KYMGRTSFD (position 81-89, hB7-2) (SEQ ID NO:17) or KSQDNVTELYDVS (position 188-200, hB7-2) (SEQ ID NO:18). Additional related sequences are evident from the sequence comparison and others can be inferred by considering homologous related amino acids such as aspartic acid and glutamic acid, alanine and glycine and other recognized functionally related amino acids. The B7 sequences share a highly positive charged domain with the cytoplasmic portion WKWKKKKRPRNSYKC (position 269-282, hB7-2) (SEQ ID NO:19) which is probably involved in intracellular signaling.

EXAMPLE 5

Characterization of the Recombinant B7-2 Antigen

35 A. B7-2 Binds CTLA4Ig and Not Anti-B7-1 and Anti-B7-3 Monoclonal Antibodies

COS cells transfected with either vector DNA (pCDNA1), or an expression plasmid containing B7-1 (B7-1) or B7-2 (B7-2) were prepared. After 72 hours, the transfected COS

-75-

- cells were detached by incubation in PBS containing 0.5 mM EDTA and 0.02% Na azide for 30 min. at 37°C. Cells were analyzed for cell surface expression by indirect immunofluorescence and flow cytometric analysis using fluorescein isothiocyanate conjugated (FITC) goat-anti-mouse Ig or goat-anti-human IgG FITC (Figure 9). Cell surface expression of B7-1 was detected with mAbs 133 (anti-B7-1) and BB-1 (anti-B7-1 and anti-B7-3) and with CTLA4Ig, whereas B7-2 reacted only with CTLA4Ig. Neither of the B7 transfectants showed any staining with the isotype controls (IgM or control Ig). The vector transfected COS cells showed no staining with any of the detection reagents. In addition, none of the cells showed any staining with the FITC labeled detection reagents and alone.
- 5 This demonstrates that B7-2 encodes a protein that is a CTLA4 counter-receptor but is distinct from B7-1 and B7-3.
- 10

B. RNA Blot Analysis of B7-2 Expression in Unstimulated and Activated Human B Cells, Cell Lines, and Myelomas

- 15 Human splenic B cells were isolated by removing T cells and monocytes as previously described (Freedman, A.S., Freeman, G.J., Horowitz, J.C., Daley, J., Nadler, L.M., *J. Immunol.* (1987) **137**:3260-3267). Splenic B cells were activated using anti-Ig beads and cells were harvested at the indicated times (Freedman et al., (1987), cited supra). Human myelomas from bone marrow specimens were enriched by removing T cells and monocytes using E rosettes and adherence as previously described (Freeman, G.J., et al., *J. Immunol.* (1989) **143**:2714-2722). RNA was prepared by guanidine thiocyanate homogenization and cesium chloride centrifugation. Equal amounts of RNA (20 μ g) were electrophoresed on an agarose gel, blotted, and hybridized to 32 P-labelled B7-2 cDNA. Figure 10, panel a, shows RNA blot analysis of unstimulated and anti-Ig activated human splenic B cells and of cell lines including Raji (B cell Burkitts lymphoma), Daudi (B cell Burkitt's lymphoma), RPMI 8226 (myeloma), K562 (erythroleukemia), and REX (T cell acute lymphoblastic leukemia). Figure 10, panel b shows RNA blot analysis of human myeloma specimens.
- 20
- 25

Three mRNA transcripts of 1.35, 1.65 and 3.0 kb were identified by hybridization to the B7-2 cDNA (Figure 10, panel b). RNA blot analysis demonstrated that B7-2 mRNA is expressed in unstimulated human splenic B cells and increases 4-fold following activation (Figure 10, panel a). B7-2 mRNA was expressed in B cell neoplastic lines (Raji, Daudi) and a myeloma (RPMI 8226) but not in the erythroleukemia K562 and the T cell line REX. In contrast, we have previously shown that B7-1 mRNA is not expressed in resting B cells and is transiently expressed following activation (G.J. Freeman et al. (1989) supra). Examination of mRNA isolated from human myelomas demonstrates that B7-2 mRNA is expressed in 6 of 6 patients, whereas B7-1 was found in only 1 of these 6 (G.J. Freeman et al. (1989) supra). Thus, B7-1 and B7-2 expression appears to be independently regulated.

30

35

C. Costimulation

Human CD28⁺ T cells were isolated by immunomagnetic bead depletion using monoclonal antibodies directed against B cells, natural killer cells and macrophages as previously described (Gimmi, C.D., et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6586-6590). B7-1, B7-2 and vector transfected COS cells were harvested 72 hours after transfection, incubated with 25 µg/ml of mitomycin-C for 1 hour, and then extensively washed. 10⁵ CD28⁺ and T cells were incubated with 1 ng/ml of phorbol myristic acid (PMA) and the indicated number of COS transfectants (Figure 11). As shown in Figure 11, panel a, T cell proliferation was measured by ³H-thymidine (1 µCi) incorporated for the last 12 hours of a 72 hour incubation. Panel b of Figure 11 shows IL-2 production by T cells as measured by ELISA (Biosource, CA) using supernatants harvested 24 hours after the initiation of culture.

D. B7-2 Costimulation is not Blocked by Anti-B7-1 and Anti-B7-3 mAbs but is Blocked by CTLA4-Ig and Anti-CD28 Fab

Human CD28⁺ T cells were isolated by immunomagnetic bead depletion using mAbs directed against B cells, natural killer cells, and macrophages as previously described (Gimmi, C.D., Freeman, G.J., Gribben, J.G., Gray, G., Nadler, L.M. (1993) *Proc. Natl. Acad. Sci USA* **90**, 6586-6590). B7-1, B7-2, and vector transfected COS cells were harvested 72 hours after transfection, incubated with 25 µg/ml of mitomycin-C for 1 hour, and then extensively washed. 10⁵ CD28⁺ T cells were incubated with 1 ng/ml of phorbol myristic acetate (PMA) and 2 x 10⁴ COS transfectants. Blocking agents (10 µg/ml) are indicated on the left side of Figure 12 and include: 1) no monoclonal antibody (no blocking agents), 2) mAb 133 (anti-B7-1 mAb), 3) mAb BB1 (anti-B7-1 and anti-B7-3 mAb), 4) mAb B5 (control IgM mAb), 5) anti-CD28 Fab (mAb 9.3), 6) CTLA-Ig, and 7) control Ig. Panel a of Figure 12 shows proliferation measured by ³H-thymidine (1 µCi) incorporation for the last 12 hours of a 72 hour incubation. Figure 12, panel b, shows IL-2 production as measured by ELISA (Biosource, CA) using supernatants harvested 24 hours after the initiation of culture.

B7-1 and B7-2 transfected COS cells costimulated equivalent levels of T cell proliferation when tested at various stimulator to responder ratios (Figure 11). Like B7-1, B7-2 transfected COS cell costimulation resulted in the production of IL-2 over a wide range of stimulator to responder cell ratios (Figure 11). In contrast, vector transfected COS cells did not costimulate T cell proliferation or IL-2 production.

-77-

E. B7-2 Costimulation is not Blocked by Anti-B7-1 and Anti-B7-3 mAbs but is Blocked by CTLA4-Ig and Anti-CD28 Fab

- Human CD28⁺ T cells were isolated by immunomagnetic bead depletion using mAbs directed against B cells, natural killer cells, and macrophages as previously described
- 5 (Gimmi, C.D., Freeman, G.J., Gribben, J.G., Gray, G., Nadler, L.M. (1993) *Proc. Natl. Acad. Sci USA* **90**, 6586-6590). B7-1, B7-2, and vector transfected COS cells were harvested 72 hours after transfection, incubated with 25 μ g/ml of mitomycin-C for 1 hour, and then extensively washed. 10⁵ CD28⁺ T cells were incubated with 1 ng/ml of phorbol myristic acetate (PMA) and 2 x 10⁴ COS transfectants. Blocking agents (10 μ g/ml) are indicated on
10 the left side of Figure 12 and include: 1) no monoclonal antibody (no blocking agents), 2) mAb 133 (anti-B7-1 mAb), 3) mAb BB1 (anti-B7-1 and anti-B7-3 mAb), 4) mAb B5 (control IgM mAb), 5) anti-CD28 Fab (mAb 9.3), 6) CTLA-Ig, and 7) control Ig. Panel a of Figure 12 shows proliferation measured by ³H-thymidine (1 μ Ci) incorporation for the last 12 hours of a 72 hour incubation. Figure 12, panel b, shows IL-2 production as measured by
15 ELISA (Biosource, CA) using supernatants harvested 24 hours after the initiation of culture.

To distinguish B7-2 from B7-1 and B7-3, mAbs directed against B7-1 and B7-3 were used to inhibit proliferation and IL-2 production of submitogenically activated human CD28⁺ T cells. Both B7-1 and B7-2 COS transfectants costimulated T cell proliferation and IL-2 production (Figure 12). MAbs 133 (Freedman, A.S. et al. (1987) *supra*) (anti-B7-1) and BB1 (Boussiotis, V.A., et al., (in review) *Proc. Natl. Acad. Sci. USA*; Yokochi, T., Holly, R.D., Clark, E.A. (1982) *J. Immunol.* **128**, 823-827) (anti-B7-1 and anti-B7-3) completely inhibited proliferation and IL-2 secretion induced by B7-1 but had no effect upon costimulation by B7-2 transfected COS cells. Isotype matched control B5 mAb had no effect. To determine whether B7-2 signals via the CD28/CTLA4 pathway, anti-CD28 Fab and CTLA4-Ig fusion protein were tested to determine whether they inhibited B7-2 costimulation. Both anti-CD28
20 Fab and CTLA4-Ig inhibited proliferation and IL-2 production induced by either B7-1 or B7-2 COS transfectants whereas control Ig fusion protein had no effect (Figure 12). While CTLA4-Ig inhibited B7-2 costimulation of proliferation by only 90%, in other experiments inhibition was more pronounced (98-100%). None of the blocking agents inhibited T cell
25 proliferation or IL-2 production induced by the combination of PMA and phytohemagglutinin.

Like B7-1, B7-2 is a counter-receptor for the CD28 and CTLA4 T cell surface molecules. Both proteins are similar in that they are: 1) expressed on the surface of APCs; 2) structurally related to the Ig supergene family with an IgV and IgC domain which share
35 26% amino acid identity, and 3) capable of costimulating T cells to produce IL-2 and proliferate. However, B7-1 and B7-2 differ in several fundamental ways. First, B7-2 mRNA is constitutively expressed in unstimulated B cells, whereas B7-1 mRNA does not appear

-78-

until 4 hours and cell surface protein is not detected until 24 hours (Freedman, A.S., et al. (1987) *supra*; Freeman, G.J., et al. (1989) *supra*). Unstimulated human B cells do not express CTLA4 counter-receptors on the cell surface and do not costimulate T cell proliferation (Boussiotis, V.A., et al. *supra*). Therefore, expression of B7-2 mRNA in unstimulated B cells 5 would allow rapid expression of B7-2 protein on the cell surface following activation, presumably from stored mRNA or protein. Costimulation by B7-2 transfectants is partially sensitive to paraformaldehyde fixation, whereas B7-2 costimulation is resistant (Gimmi, C.D., et al. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6575-6579). Second, expression of B7-1 and B7-2 in cell lines and human B cell neoplasms substantially differs. Third, B7-2 protein 10 contains a longer cytoplasmic domain than B7-1 and this could play a role in signaling B-cell differentiation. These phenotypic and functional differences suggest that these homologous molecules may have biologically distinct functions.

EXAMPLE 6

15 Cloning and Sequencing of the Murine B7-2 Antigen

A. Construction of cDNA Library

16 A cDNA library was constructed in the pCDM8 vector (Seed, *Nature*, 329:840 (1987)) using poly (A)⁺ RNA from dibutryl cyclic AMP (cAMP) activated M12 cells (a murine B cell tumor line) as described (Aruffo et al, *Proc. Natl. Acad. Sci. USA*, 84:3365 20 (1987)).

17 M12 cells were cultured at 1×10^6 cells/ml in complete culture media, {RPMI 1640 with 10% heat inactivated fetal calf serum (FCS), 2mM glutamine, 1 mM sodium pyruvate, penicillin (100 units/ml), streptomycin sulfate (100 μ g/ml) and gentamycin sulfate (5 μ g/ml)}, 25 in tissue culture flasks and were activated by 300 μ g/ml dibutryl cAMP (Nabavi, N., et al. (1992) *Nature* **360**, 266-268). Activated M12 cells were harvested after 0, 6, 12, 18, 24 and 30 hours.

18 RNA was prepared by homogenizing activated M12 cells in a solution of 4M guanidine thiocyanate, 0.5% sarkosyl, 25mM EDTA, pH 7.5, 0.13% Sigma anti-foam A, and 30 0.7% mercaptoethanol. RNA was purified from the homogenate by centrifugation for 24 hour at 32,000 rpm through a solution of 5.7M CsCl, 10mM EDTA, 25mM Na acetate, pH 7. The pellet of RNA was dissolved in 5% sarkosyl, 1mM EDTA, 10mM Tris, pH 7.5 and extracted with two volumes of 50% phenol, 49% chloroform, 1% isoamyl alcohol. RNA was ethanol precipitated twice. Poly (A)⁺ RNA used in cDNA library construction was purified 35 by two cycles of oligo (dT)-cellulose selection.

Complementary DNA was synthesized from 5.5 μ g of dibutryl cAMP activated murine M12 cell poly(A)⁺ RNA in a reaction containing 50mM Tris, pH 8.3, 75mM KCl,

-79-

- 3mM MgCl₂, 10mM dithiothreitol, 500μM dATP, dCTP, dGTP, dTTP, 50μg/ml oligo(dT)12-18, 180 units/ml RNasin, and 10,000 units/ml Moloney-MLV reverse transcriptase in a total volume of 55μl at 37°C for 1 hr. Following reverse transcription, the cDNA was converted to double-stranded DNA by adjusting the solution to 25mM Tris, pH 8.3, 100mM KCl, 5mM MgCl₂, 250μM each dATP, dCTP, dGTP, dTTP, 5mM dithiothreitol, 250 units/ml DNA polymerase I, 8.5 units/ml ribonuclease H and incubating at 16°C for 2 hr. EDTA was added to 18mM and the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate and with 4 micrograms of linear polyacrylamide as carrier. Following reverse transcription, the reverse transcriptase was inactivated by heating at 70°C for 10 min. The cDNA was converted to double-stranded DNA by adding 320μl H₂O and 80μl of a solution of 0.1M Tris, pH 7.5, 25mM MgCl₂, 0.5M KCl, 250μg/ml bovine serum albumin, and 50mM dithiothreitol, and adjusting the solution to 200μM each dATP, dCTP, dGTP, dTTP, 50 units/ml DNA polymerase I, 8 units/ml ribonuclease H and incubating at 16°C for 2 hours. EDTA was added to 18 mM and the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate and with 4 micrograms of linear polyacrylamide as carrier.
- 2μg of non-selfcomplementary BstXI adaptors were added to the DNA as follows:
- 20 The double-stranded cDNA from 5.5μg of poly(A)⁺ RNA was incubated with 3.6μg of a kinased oligonucleotide of the sequence CTTTAGAGCACA (SEQ ID NO:15) and 2.4μg of a kinased oligonucleotide of the sequence CTCTAAAG (SEQ ID NO:16) in a solution containing 6mM Tris, pH 7.5, 6mM MgCl₂, 5mM NaCl, 350μg/ml bovine serum albumin, 7mM mercaptoethanol, 0.1mM ATP, 2mM dithiothreitol, 1mM spermidine, and 600 units T4
- 25 DNA ligase in a total volume of 0.45ml at 15° for 16 hours. EDTA was added to 34mM and the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate.
- DNA larger than 600bp was selected as follows: The adaptored DNA was redissolved
- 30 in 10mM Tris, pH 8, 1mM EDTA, 600mM NaCl, 0.1% sarkosyl and chromatographed on a Sepharose CL-4B column in the same buffer. DNA in the void volume of the column (containing DNA greater than 600bp) was pooled and ethanol precipitated.
- The pCDM8 vector was prepared for cDNA cloning by digestion with BstXI and purification on an agarose gel. Adaptored DNA from 5.5μg of poly(A)⁺RNA was ligated to
- 35 2.25μg of BstXI cut pCDM8 in a solution containing 6mM Tris, pH 7.5, 6mM MgCl₂, 5mM NaCl, 350μg/ml bovine serum albumin, 7mM mercaptoethanol, 0.1mM ATP, 2mM dithiothreitol, 1mM spermidine, and 600 units T4 DNA ligase in a total volume of 1.5ml at

-80-

15° for 24 hr. The ligation reaction mixture was transformed into competent *E.coli* MC1061/P3 and a total of 200 x 10⁶ independent cDNA clones were obtained.

Plasmid DNA was prepared from a 500 ml culture of the original transformation of the cDNA library. Plasmid DNA was purified by the alkaline lysis procedure followed by 5 twice banding in CsCl equilibrium gradients (Maniatis et al, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1987)).

B. Cloning Procedure

- In the first round of screening, thirty 100 mm dishes of 50% confluent COS cells were 10 transfected with 0.05µg/ml activated M12 murine B cell library DNA using the DEAE-Dextran method (Seed et al, *Proc. Natl. Acad. Sci. USA*, 84:3365 (1987)). The cells were trypsinized and re-plated after 24 hours. After 47 hours, the cells were detached by incubation in PBS/0.5 mM EDTA, pH 7.4/0.02% Na azide at 37°C for 30 min. The detached cells were treated with 10 µg/ml/human CTLA4Ig and murine CD28Ig for 45 minutes at 4°C. 15 Cells were washed and distributed into panning dishes coated with affinity-purified Goat anti-human IgG antibody and allowed to attach at room temperature. After 3 hours, the plates were gently washed twice with PBS/0.5mM EDTA, pH 7.4/0.02% Na azide, 5% FCS and once with 0.15M NaCl, 0.01 M Hepes, pH 7.4, 5% FCS. Episomal DNA was recovered from the panned cells and transformed into *E. coli* DH10B/P3. The plasmid DNA was re- 20 introduced into COS cells via spheroplast fusion as described (Seed et al, *Proc. Natl. Acad. Sci. USA*, 84:3365 (1987)) and the cycle of expression and panning was repeated twice. In the second and third rounds of selection, after 47 hours, the detached COS cells were first incubated with α-murine B7-1 mAb (16-10A1, 10 µg/ml), and COS cells expressing B7-1 were removed by α-mouse IgG and IgM coated magnetic beads. COS cells were then treated 25 with 10µg/ml of human CTLA4Ig and murine CD28Ig and murine B7-2 expressing COS cells were selected by panning on dishes coated with goat anti-human IgG antibody. After the third round, plasmid DNA was prepared from individual colonies and transfected into COS cells by the DEAE-Dextran method. Expression of B7-2 on transfected COS cells was analyzed by indirect immunofluorescence with CTLA4Ig.
- 30 After the final round of selection, plasmid DNA was prepared from individual colonies. A total of 6 of 8 candidate clones contained a cDNA insert of approximately 1.2 kb. Plasmid DNA from these eight clones was transfected into COS cells. All six clones with the 1.2 Kb cDNA insert were strongly positive for B7-2 expression by indirect immunofluorescence using CTLA4Ig and flow cytometric analysis.

-81-

C. Sequencing

The B7-2 cDNA insert in clone4 was sequenced in the pCDM8 expression vector employing the following strategy. Initial sequencing was performed using sequencing primers T7, CDM8R (Invitrogen) homologous to pCDM8 vector sequences adjacent to the 5 cloned B7-2 cDNA (see Table II). Sequencing was performed using dye terminator chemistry and an ABI automated DNA sequencer. (ABI, Foster City, CA). DNA sequence obtained using these primers was used to design additional sequencing primers (see Table II). This cycle of sequencing and selection of additional primers was continued until the murine B7-2 cDNA was completely sequenced on both strands.

10

TABLE II

T7(F) (SEQ ID NO:3)	5'd[TAATACGACTCACTATAGGG]3'
CDM8(R) (SEQ ID NO:4)	5'd[TAAAGGTTCCCTCACAAAG]3'
15 MBX4-1F (SEQ ID NO:24)	5'd[ACATAAGCCTGAGTGAGCTGG]3'
MBX4-2R (SEQ ID NO:25)	5'd[ATGATGAGCAGCATCACAAAGG]3'
MBX4-14 (SEQ ID NO:26)	5'd[TGGTCGAGTGAGTCCGAATAC]3'
MBX4-2F (SEQ ID NO:27)	5'd[GACGAGTAGTAACATACAGTG]3'
20	A murine B7-2 clone (mB7-2, clone 4) was obtained containing an insert of 1,163 base pairs with a single long open reading frame of 927 nucleotides and approximately 126 nucleotides of 3' noncoding sequences (Figure 14, SEQ ID NO:22). The predicted amino acid sequence encoded by the open reading frame of the protein is shown below the nucleotide sequence in Figure 14. The encoded murine B7-2 protein, is predicted to be 309
25	amino acid residues in length (SEQ ID NO:23). This protein sequence exhibits many features common to other type I Ig superfamily membrane proteins. Protein translation is predicted to begin at the methionine codon (ATG, nucleotides 111 to 113) based on the DNA homology in this region with the consensus eucaryotic translation initiation site (see Kozak, M. (1987) <i>Nucl. Acids Res.</i> 15 :8125-8148). The amino terminus of the murine B7-2 protein
30	(amino acids 1 to 23) has the characteristics of a secretory signal peptide with a predicted cleavage between the alanine at position 23 and the valine at position 24 (von Heijne (1987) <i>Nucl. Acids Res.</i> 14 :4683). Processing at this site would result in a murine B7-2 membrane bound protein of 286 amino acids having an unmodified molecular weight of approximately 32 kDa. This protein would consist of an approximate extracellular Ig superfamily V and C
35	like domains of from about amino acid residue 24 to 246, a hydrophobic transmembrane domain of from about amino acid residue 247 to 265, and a long cytoplasmic domain of from about amino acid residue 266 to 309. The homologies to the Ig superfamily are due to the

-82-

two contiguous Ig-like domains in the extracellular region bound by the cysteines at positions 40 to 110 and 157 to 216. The extracellular domain also contains nine potential N-linked glycosylation sites and, like murine B7-1, is probably glycosylated. Glycosylation of the murine B7-2 protein may increase the molecular weight to about 50-70 kDa. The 5 cytoplasmic domain of murine B7-2 contains a common region which has a cysteine followed by positively charged amino acids which presumably functions as signaling or regulatory domain within an APC. Comparison of both the nucleotide and amino acid sequences of murine B7-2 with the GenBank and EMBL databases yielded significant homology (about 26% amino acid sequence identity) with human and murine B7-1. Murine 10 B7-2 exhibits about 50% identity and 67% similarity with its human homologue, hB7-2. *E. coli* (DH106/p3) transfected with a vector (plasmid pmBx4) containing a cDNA insert encoding murine B7-2 (clone 4) was deposited with the American Type Culture Collection (ATCC) on August 18, 1993 as Accession No. 69388.

15 D. Costimulation

CD4⁺ murine T cells were purified by first depleting red blood cells by treatment with Tris-NH₄Cl. T cells were enriched by passage over a nylon wool column. CD4⁺ T cells were purified by two-fold treatment with a mixture of anti-MHC class II and anti-CD28 mAbs and rabbit complement. Murine B7-1 (obtained from Dr. Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA; see also, Freeman, G.J. et al (1991) *J. Exp. Med.* **174**, 625-631) murine B7-2, and vector transfected COS cells were harvested 72 hours after transfection, incubated with 25μg/ml mitomycin-C for one hour, and then extensively washed. 10⁵ murine CD4⁺ T cells were incubated with 1ng/ml of phorbol myristic acid (PMA) and 2 x 10⁴ COS transfectants (Table III). T cell proliferation was measured by ³H-thymidine (1μCi) incorporated for the last 12 hours of a 72 hour incubation.

TABLE III
³H-Thymidine Incorporation (cpm)

30	CD4 ⁺ T cells	175
	CD4 ⁺ T cells + 1ng/ml PMA	49
	CD4 ⁺ T cells + COS-vector	1750
	CD4 ⁺ T cells + COS-B7-1	4400
	CD4 ⁺ T cells + COS-B7-2	2236
35	CD4 ⁺ T cells + 1ng/ml PMA + COS-vector	2354
	CD4 ⁺ T cells + 1ng/ml PMA + COS-B7-1	67935
	CD4 ⁺ T cells + 1ng/ml PMA + COS-B7-2	43847

EXAMPLE 7**Construction and Characterization of Human B7-2 Immunoglobulin Fusion Proteins****5 A. Preparation Of Human B7-2Ig Fusion Proteins**

The extracellular portion of human B7-2 was prepared as a fusion protein coupled to an immunoglobulin constant region. The immunoglobulin constant region may contain genetic modifications including those which reduce or eliminate effector activity inherent in the immunoglobulin structure. Briefly, DNA encoding the extracellular portion of hB7-2 was joined to DNA encoding the hinge, CH2 and CH3 regions of human IgCy1 or IgCy4 modified by directed mutagenesis. This was accomplished as described in the following subsections.

B. Preparation of Gene Fusions

DNA fragments corresponding to the DNA sequences of interest were prepared by polymerase chain reaction (PCR) using primer pairs described below. In general, PCR reactions were prepared in 100 µl final volume composed of *Taq*, polymerase buffer (Gene Amp PCR Kit, Perkin-Elmer/Cetus, Norwalk, CT) containing primers (1 µM each), dNTPs (200 µM each) 1 ng of template DNA, and *Taq*, polymerase (Saiki, R.K., et al. (1988) *Science* 239:487-491). PCR DNA amplifications were run on a thermocycler (Ericomp, San Diego, CA) for 25 to 30 cycles each composed of a denaturation step (1 minute at 94°C), a renaturation step (30 seconds at 54°C), and a chain elongation step (1 minute at 72°C). The structure of each hB7-2 Ig genetic fusion consisted of a signal sequence to facilitate secretion coupled to the extracellular domain of B7-2 and the hinge, CH2 and CH3 domains of human IgCy1 or IgCy4. The IgC gamma 1 and IgC gamma 4 sequences contained nucleotide changes within the hinge region to replace cysteine residues available for disulfide bond formation with serine residues and may contain nucleotide changes to replace amino acids within the CH2 domain thought to be required for IgC binding to Fc receptors and complement activation.

Sequence analysis confirmed structures of both my₄ and γ₁ clones, and each construct was used to transfect 293 cells to test transient expression. hIgG ELISA measured/confirmed transient expression levels approximately equal to 100 ng protein/ml cell supernatant for both constructs. NSO cell lines were transfected for permanent expression the the fusion proteins.

C. Genetic Construction of hB7-2Ig Fusion Proteins**35 (1). Preparation of Signal Sequence**

PCR amplification was used to generate an immunoglobulin signal sequence suitable for secretion of the B7-2Ig fusion protein from mammalian cells. The Ig signal sequence was

-84-

prepared from a plasmid containing the murine IgG heavy chain gene (Orlandi, R. et al. (1989) *Proc. Natl. Acad. Sci. USA.* 86:38333837) using the oligonucleotide 5'-GGCACTAGGTCTCCAGCTTGAGATCACAGTCTCTCTAC-3' (#01) (SEQ ID NO:) as the forward primer and the oligonucleotide 5'-
5 GCTTGAATCTTCAGAGGAGCGGAGTGGACACCTGTGG-3' (#02) (SEQ ID NO:) as the reverse PCR primer. The forward PCR primer (SEQ ID NO:) contains recognition sequences for restriction enzymes Bsal and is homologous to sequences 5' to the initiating methionine of the Ig signal sequence. The reverse PCR primer (SEQ ID NO:) is composed of sequences derived from the 5' end of the extracellular domain of hB7-2 and the 3' end of
10 the Ig signal sequence. PCR amplification of the murine Ig signal template DNA using these primers resulted in a 224 bp product which is composed of BsaI restriction sites followed by the sequence of the Ig signal region fused to the first 20 nucleotides of the coding sequence of the extracellular domain of hB7-2. The junction between the signal sequence and hB7-2 is such that protein translation beginning at the signal sequence will continue into and through
15 hB7-2 in the correct reading frame.

(2). Preparation of the hB7-2 Gene Segment

The extracellular domain of the hB7.2 gene was prepared by PCR amplification of plasmid containing the hB7-2 cDNA inserted into expression vector pCDNA1 (Freeman et al., *Science* 262:909-11 (1994)):

The extracellular domain of hB7-2 was prepared by PCR amplification using oligonucleotide 5'-GCTCCTCTGAAGATTCAAGC-3' (#03) (SEQ ID NO:) as the forward primer and oligonucleotide 5'-GGCACTATGATCAGGGGGAGGCTGAGGTCC-3' (#04) (SEQ ID NO:) as the reverse primer. The forward PCR primer contained sequences corresponding to the first 20 nucleotides of the B7-2 extracellular domain and the reverse PCR primer contained sequences corresponding to the last 22 nucleotides of the B7-2 extracellular domain followed by a Bcl I restriction site and 7 noncoding nucleotides. PCR amplification with primer #03 and #04 yields a 673 bp product corresponding to the extracellular IgV and IgC like domains of hB7-2 followed by a unique Bcl I restriction site.
25 The signal sequence was attached to the extracellular portion of hB7-2 by PCR as follows. DNA-PCR products obtained above corresponding to the signal sequence and the hB7-2 extracellular domain were mixed in equimolar amounts, denatured by heating to 100°C, held at 54°C for 30°C to allow the complementary ends to anneal and the strands were filled in using dNTPs and Toq polymerase. PCR primers #01 and #04 were added and
30 the entire fragment produced by PCR amplification to yield a ~880 fragment composed of a BsaI restriction site followed by the signal sequence fused to the extracellular domain of hB7-2, followed by a Bcl I restriction site.

(3) Cloning and Modification of Immunoglobulin Fusion Domain

Plasmid pSP721gG1 was prepared by cloning the 2000 bp segment of human IgG1 heavy chain genomic DNA (Ellison, J.W., et al. (1982) *Nucl. Acids. Res.* 10:4071-4079) into the multiple cloning site of cloning vector pSP72 (Promega, Madison, WI). Plasmid pSP721gG1 contained genomic DNA encoding the CH1, hinge, CH2 and CH3 domains of the heavy chain human IgCyl gene. PCR primers designed to amplify the hinge-CH2-CH3 portion of the heavy chain along with the intervening DNA were prepared as follows. The forward PCR primer 5'-GCATTTAAGCTTTCTGATCAGGAGCCAAATCTTCT 5 GACAAAACACTCACACATCTCCACCGTCTCCAGGTAAGCC-3' (SEQ ID NO:) contained HindIII and Bcl I restriction sites and was homologous to the hinge domain sequence except for five nucleotide substitutions which would change the three cysteine residues to serines. The reverse PCR primer 5'TAATACGACTCACTATAGGG-3' (SEQ ID NO:) was identical to the commercially available T7 primer (Promega, Madison, WI).

10 Amplification with these primers yielded a 1050 bp fragment bounded on the 5' end by HindIII and BcI I restriction sites and on the 3' end by BamH1, SmaI, KpnI, SacI, EcoR1, Clal, EcoR5 and BglII restriction sites. This fragment contained the IgC hinge domain in which the three cysteine codons had been replaced by serine codons followed by an intron, the CH2 domain, an intron, the CH3 domain and additional 3' sequences. After PCR 15 amplification, the DNA fragment was digested with HindIII and EcoR1 and cloned into expression vector pNRDSH digested with the same restriction enzymes. This created plasmid pNRDSH/IgG1.

20

A similar PCR based strategy was used to clone the hinge-CH2-CH3 domains of human IgCgamma4 constant regions. A plasmid, p428D (Medical Research Council, 25 London, England) containing the complete IgCgamma4 heavy chain genomic sequence (Ellison, J. Buxbaum, J. and Hood, L.E. (1981) *DNA* 1: 11 -18) was used as a template for PCR amplification using oligonucleotide 5'GAGCATTTCTGATCAGGA GTCCAAATATGGTCCCCATCCCATCATCCCCAGGTAAGCCAACCC-3' (SEQ ID NO:) as the forward PCR primer and oligonucleotide 30 5'GCAGAGGAATCGAGCTCGGTACCCGGGATCCCCAGTGTGGGGACAGTGGGA CCGCTCTGCCCTCCC-3' (SEQ ID NO:) as the reverse PCR primer. The forward PCR primer (SEQ ID NO:) contains a BcI I restriction site followed by the coding sequence for the hinge domain of IgCgamma4. Nucleotide substitutions have been made in the hinge region to replace the cysteines residues with serines. The reverse PCR primer (SEQ ID NO.) 35 contains a PspAI restriction site (5'CCCGGG-3'). PCR amplification with these primers results in a 1179 bp DNA fragment. The PCR product was digested with BcI I and PspAI and ligated to pNRDSH/IgG1 digested with the same restriction enzymes to yield plasmid

-86-

pNRDSH/IgG4. In this reaction, the IgC γ 4 domain replaced the IgC γ 1 domain present in pNRDSH/IgG1.

Modification of the CH2 domain in IgC to replace amino acids thought to be involved in binding to Fc receptor was accomplished as follows. Plasmid pNRDSH/IgG1 served as template for modifications of the IgC γ 1 CH2 domain and plasmid pNRDSH/IgG4 served as template for modifications of the IgC γ 4 CH2 domain. Plasmid pNRDSH/IgG1 was PCR amplified using a forward PCR primer (SEQ ID NO:) and oligonucleotide 5'-GGGTTTT GGGGGGAAGAGGAAGACTGACGGTGCCCCC TCGGCTTCAGGTGCTGAGGAAG-3' (SEQ ID NO:) as the reverse PCR primer. The forward PCR primer (SEQ ID NO:) has been previously described and the reverse PCR primer (SEQ ID NO:) was homologous to the amino terminal portion of the CH2 domain of IgG1 except for five nucleotide substitutions designed to change amino acids 234, 235, and 237 (Canfield, S. M. and Morrison, S. L. (1991) *J. Exp. Med.* 173: 1483-1491.) from Leu to Ala, Leu to Glu, and Gly to Ala, respectively. Amplification with these PCR primers will yield a 239 bp DNA fragment consisting of a modified hinge domain, an intron and modified portion of the CH2 domain. Plasmid pNRDSH/IgG1 was also PCR amplified with the oligonucleotide 5'- CATCTCTTCCTCAGCACCTGAAGCCGAGGGGGCACCGTCAGTCTTCCTCTCCC CC-3' (SEQ ID NO:) as the forward primer and oligonucleotide (SEQ ID NO:) as the reverse PCR primer. The forward PCR primer (SEQ ID NO:) is complementary to primer (SEQ ID NO:) and contains the five complementary nucleotide changes necessary for the CH2 amino acid replacements. The reverse PCR primer (SEQ ID NO:) has been previously described. Amplification with these primes yields a 875 bp fragment consisting of the modified portion of the CH2 domain, an intron, the CH3 domain, and 3' additional sequences. The complete IgC γ 1 segment consisting of modified hinge domain, modified CH2 domain and CH3 domain was prepared by an additional PCR reaction. The purified products of the two PCR reactions above were mixed, denatured (95°C, 1 minute) and then renatured (54°C, 30 seconds) to allow complementary ends of the two fragments to anneal. The strands were filled in using dNTP and Taq polymerase and the entire fragment amplified using forward PCR primer (SEQ ID NO:) and reverse PCR primer (SEQ ID NO:). The resulting fragment of 1050 bp was purified, digested with HindIII and EcoR1 and ligated to pNRDSH previously digested with the same restriction enzymes to yield plasmid pNRDSHIgG1 m.

Two amino acids at immunoglobulin positions 235 and 237 were changed from Leu to Glu and Gly to Ala, respectively, within the IgC γ 4 CH2 domain to eliminate Fc receptor binding. Plasmid pNRDSH/IgG4 was PCR amplified using the forward primer (SEQ ID NO:) and the oligonucleotide 5'- CGCACGTGACCTCAGGGGTCCGGGAGATCATGAGAGTGTCTGGGTTTGGGG GGAACAGGAAGACTGATGGTGCCCCCTCGAACTCAGGTGCTGAGG-3' (SEQ ID

-87-

NO:) as the reverse primer. The forward primer has been previously described and the reverse primer was homologous to the amino terminal portion of the CH2 domain, except for three nucleotide substitutions designed to replace the amino acids described above. This primer also contained a PmlI restriction site for subsequent cloning. Amplification with these 5 primers yields a 265 bp fragment composed of the modified hinge region, and intron, and the modified 5' portion of the CH2 domain.

Plasmid pNRDSH/IgG4 was also PCR amplified with the oligonucleotide 5
'-CCTCAGCACCTGAGTCGAGGGGCACCATCAGTCTCCTGTTCCCCC
AAAACCCAAGGACACTCTCATGATCTCCGGACCCCTGAGGTACGTGCG-3'
10 (SEQ ID NO:) as the forward primer and oligonucleotide (SEQ ID NO:) as the reverse PCR primer. The forward PCR primer (SEQ ID NO:) is complementary to primer (SEQ ID NO:) and contains the three complementary nucleotide changes necessary for the CH2 amino acid replacements. The reverse PCR primer (SEQ ID NO:) has been previously described. Amplification with these primes yields a 1012 bp fragment consisting of the modified portion 15 of the CH2 domain, an intron, the CH3 domain, and 3' additional sequences. The complete IgCy4 segment consisting of modified hinge domain, modified CH2 domain and CH3 domain was prepared by an additional PCR reaction. The purified products of the two PCR reactions above were mixed, denatured (95°C, 1 minute) and then renatured (54°C, 30 seconds) to allow complementary ends of the two fragments to anneal. The strands were filled in using dNTP 20 and Taq polymerase and the entire fragment amplified using forward PCR primer (SEQ ID NO:) and reverse PCR primer (SEQ ID NO:). The resulting fragment of 1179 bp was purified, digested with BclI and PspAI and ligated to pNRDSH previously digested with the same restriction enzymes to yield plasmid pNRDSH/IgG4m.

25 (4) Assembly of Final hB7-2Ig Genes

The PCR fragment corresponding to the Ig signal-hB7-2 gene fusion prepared above was digested with BsaI and Bc11 restriction enzymes and ligated to pNRDSH/IgG1, pNRDSH/IgG1m, pNRDSH/IgG4, and pNRDSH/IgG4m previously digested with Hind III and BclI. The ligated plasmids were transformed into *E. coli* JM109 using CaCl₂ competent 30 cells and transformants were selected on L-agar containing ampicillin (50 µg/ml; Molecular Cloning: A Laboratory Manual (1982) Eds. Maniatis, T., Fritsch, E. E., and Sambrook, J. Cold Spring Harbor Laboratory). Plasmids isolated from the transformed *E. coli* were analyzed by restriction enzyme digestion. Plasmids with the expected restriction plasmid were sequenced to verify all portions of the signal-hB7-2-IgG gene fusion segments.

-88-

D. Expression Cloning of hB7-2V-IgG1 and hB7-2C IgG1

The variable and constant domains of human B7-2 were separately cloned into pNRDSH/IgG1. These clonings were accomplished using PCR. The portions of hB7-2 corresponding to the variable and constant regions were determined from intron/exon
5 mapping and previously published gene structure analysis.

Human B7-2 Variable Domain

5'GCTCCTCTGAAGATT..... GAACTGTCAGTGCTT3' (SEQ ID NO:)
A P L K I E L S V L (SEQ ID NO:)

10

Human B7-2 Constant Domain

5'GCTAACTTCAGTCAA..... CCTTTCTCTATAGAG3' (SEQ ID NO:)
A N F S Q P F S I E (SEQ ID NO:)

15

(1) Assembly of hB7-2V Ig

The hB7-2V domain Ig sequence was assembled using a PCR strategy similar to that shown above. The signal sequence was derived from the onco M gene by PCR amplification of a plasmid containing the onco M gene using oligonucleotide 5'-GCAACCGGAAGCTTGCCACCATGGGGTACTGCTCACACAGAGGACG-3' (#05) (SEQ ID NO:) as the forward PCR primer and 5'-AGTCTCATTGAAATAAGCTTGAATCTTCAGAGGAGCCATGCTGCCATGCTTGGAAACAGGAG-3' (#06) (SWQ ID NO:) as the reverse primer. The forward PCR primer (#05) contains a Hind III restriction site and the amino terminal portion of the onco M signal sequence. The reverse PCR (#06) contains the sequence corresponding to the 3' portion of the onco M signal sequence fused to the 5' end of the hB7-2 IgV like domain.
20
25

The hB7-2 IgV like domain was obtained by PCR amplification of the hB7-2 cDNA using oligonucleotide 5'-CTCCTGTTCCAAGCATGGCCAGCATGGCTCCTCTGAA GATTCAAGGCTTATTCAATGAGAC-3' (#07) (SEQ ID NO:) as the forward and oligonucleotide 5'-TGTGTGTGGAATTCTCATTACTGATCAAGCACTGACAGTTCAGAATTCATC-3' (#08) (SEQ ID NO :) as the reverse PCR primer. PCR amplification with these primers yields the hB7-2 IgV domain with a portion of the 3' end of the onco M signal sequence on the 5' end and a Bcl I restriction site on the 3' end. The signal and IgV domain were linked together in a PCR reaction in which equimolar amounts of the onco M signal and IgV domain DNA fragments were mixed, denatured, annealed, and the strands filled in. Subsequent PCR amplification using forward primer #05 and reverse primer #08 yielded a DNA fragment containing a Hind III restriction site, followed by the onco M signal fused to the B7-2 IgV
30
35

-89-

domain followed by a Bcl I restriction site. This PCR fragment was digested with Hind II and Bcl I and cloned into expression vector pNRDSH/IgG1 digested with the same restriction enzymes to yield pNRDSH/B7-2CIg.

5 (2). Assembly of hB7-2CIg

- The expression plasmid for hB7-2IgC domain was prepared as described above for the IgV domain except for using PCR primers specific for the IgC domain. The onco M signal sequence was prepared using oligonucleotide #05 as the forward PCR primer and oligonucleotide 5'.
- 10 AGAAATTGGTACTATTCAGGTTGACTGAAGTTAGCCATGCTGCCATGCTTGGAA
AACAGGAG-3' (#09) (SEQ ID NO:) as the reverse PCR primer. The hB7-2 IgC domain was prepared using oligonucleotide 5'.
CTCCTGTTCCAAGCATGGCCAGCATGGCTAACCTCAGTC
AACCTGAAAATAGTACCAATTTC-3' (#11) (SEQ ID NO:) as the reverse PCR primer.
15 The two PCR products were mixed and amplified with primers #05 and #11 to assemble the onco M signal sequence with the hB7-2IgC domain. The PCR product was subsequently digested with Hind III and BclI and ligated to pNRDSH/IgG1 digested with similar restriction enzymes to yield the final expression plasmid pNRDSH/hB7-2CIgG1.

20 E. Competition Binding Assays With Human B7-2Ig Fusion Proteins

- The ability of various B7 family-Ig fusion proteins to competitively inhibit the binding of biotinylated-CTLA4Ig to immobilized B7-2Ig was determined. Competition binding assays were done as follows and analysed according to McPherson (McPherson, G.A. (1985) *J. Pharmacol. Methods* 14:213-228). Soluble hCTLA4Ig was labelled with ^{125}I to a specific activity of approximately 2×10^6 cpm/pmol. hB7-2Ig fusion protein was coated overnight onto microtiter plates at $10\mu\text{g}/\text{ml}$ in 10 mM Tris-HCl, pH 8.0, 50 μl /well. The wells were blocked with binding buffer (DMEM containing 10% heat-inactivated FBS, 0.1% BSA, and 50 mM BES, pH 6.8) for 2 h at room temperature. The labeled CTLA4-Ig (4nM) was added to each well in the presence or absence of unlabeled competing Ig fusion proteins, including full-length B7-2 (hB7-2Ig), full-length B7-1 (hB7-1Ig), the variable region-like domain of B7-2 (hB7-2VIg) and the constant region-like domain of B7-2 (hB7-2CIg) and allowed to bind for 2.5 h at room temperature. The wells were washed once with ice-cold binding buffer and then four times with ice-cold PBS. Bound radioactivity was recovered by treatment of the wells with 0.5 N NaOH for 5 min and the solubilized material removed and counted in a gamma counter.

The results of these assays are shown in Figure 15 in which both hB7-2Ig (10-20 nM) and hB7-2VIg (30-40 nM) competitively inhibit the binding of CTLA4Ig to immobilized B7-

-90-

2 protein. hB7-2CIg is unable to compete with soluble CTLA4, indicating that the B7-2 binding region is found in the variable-region like domain.

F. Competitive binding Assays for B7-1 and B7-2 fusion proteins

5 The ability of the various recombinant CTLA4 forms to bind to hB7-1 or hB7-2 was assessed in a competitive binding ELISA assay as follows. Purified recombinant hB7-Ig (20 µg/ml in PBS) was bound to a Costar EIA/RIA 96 well microtiter dish (Costar Corp, Cambridge MA, USA) in 50 µL overnight at room temperature. The wells were washed three times with 200 µL of PBS and the unbound sites blocked by the addition of 1 % BSA in PBS
10 (200/well) for 1 hour at room temperature. The wells were washed as above. Biotinylated hCTLA4IgG1 (ref, MFGR; 1 µg/ml serially diluted in twofold steps to 15.6 ng/mL; 50 µL) was added to each well and incubated for 2.5 hours at room temperature. The wells were washed as above. The bound biotinylated CTLA4Ig was detected by the addition of 50 1/1 of a 1:2000 dilution of streptavidin-HRP (Pierce Chemical Co., Rockford, IL) for 30 minutes at
15 room temperature. The wells were washed as above and 50 µL of ABTS (Zymed, California) added and the developing blue color monitored at 405 nm after 30 min. A graphic representation of a typical binding assay is shown in Figure 16. The ability of the various forms of CTLA4 to compete with biotinylated CTLA4IgG1 was assessed by mixing varying amounts of the competing protein with a quantity of biotinylated CTLA4IgG1 shown to be
20 non-saturating (i.e., 70 ng/mL; 1.5nM) and performing the binding assays as described above (Figure 15). A reduction in the signal (Abs 405 nm) expected for biotinylated CTLA4IgG1 indicated a competition for binding to hB7-1.

Considering the previous evidence that CTLA4 was the high affinity receptor for B7-1, the avidity of binding of CTLA4 and CD28 to B7-1 and B7-2 was compared. B7-1-Ig or B7-2-Ig was labelled with biotin and bound to immobilized CTLA4-Ig in the presence or absence of increasing concentrations of unlabeled B7-1-Ig or B7-2-Ig. The experiment was repeated with ¹²⁵I-labeled B7-1-Ig or B7-2-Ig. Using this solid phase binding assay, the avidity of B7-2 (2.7 nM) for CTLA4 was determined to be approximately two-fold greater than that observed for B7-1 (4.6 nM). The experimentally determined IC₅₀ values are
30 indicated in the upper right corner of the panels. The affinity of both B7-1 and B7-2 for CD28 was lower and was difficult to confidently determine.

-91-

EXAMPLE 8

Production and Characterization of Monoclonal Antibodies to Human B7-2

A. Immunizations and Cell Fusions

5 Balb/c female mice (obtained from Taconic Labs, Germantown, NY) were immunized intraperitoneally with 50 µg human B7.2-Ig emulsified in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) or 10^6 CHO-human B7.2 cells per mouse. The mice were given two booster immunizations with 10-25 µg human B7.2-Ig emulsified in incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) or CHO-human B7.2 cells at
10 fourteen day intervals following the initial immunization for the next two months. The mice were bled by retro-orbital bleed and the sera assayed for the presence of antibodies reactive to the immunogen by ELISA against human B7.2-Ig. ELISA against hCTLA4-Ig was also used to control for Ig tail directed antibody responses. Mice showing a strong serological response were boosted intravenously via the tail vein with 25 µg human hB7.2-Ig diluted in phosphate-buffered saline (PBS), pH 7.2 (GIBCO, Grand Island, NY). Three to four days following this boost, the spleens from these mice were fused 5:1 with SP 2/0 myeloma cells (American Type Culture Collection, Rockville, MD, No. CRL8006), which are incapable of secreting both heavy and light immunoglobulin chains (Kearney et al. (1979) *J. Immunol.* **123**:1548). Standard methods based upon those developed by Kohler and Milstein (*Nature* (1975)
15 256:495) were used.
20 256:495) were used.

B. Antibody Screening

After 10-21 days, supernatants from wells containing hybridoma colonies from the fusion were screened for the presence of antibodies reactive to human B7.2 as follows: Each
25 well of a 96 well flat bottomed plate (Costar Corp., Cat. #3590) was coated with 50 µl per well of a 1 µg/ml human B7.2-Ig solution or 5×10^4 3T3-hB7.2 cells on lysine coated plates in phosphate-buffered saline, pH 7.2, overnight at 4 °C. The human B7.2-Ig solution was aspirated off, or the cells were cross-linked to the plates with glutaraldehyde, and the wells were washed three times with PBS, then blocked with 1% BSA solution (in PBS) (100µ
30 l/well) for one hour at room temperature. Following this blocking incubation, the wells were washed three times with PBS and 50 µl of hybridoma supernatant was added per well and incubated for 1.5 hours at room temperature. Following this incubation, the wells were washed three times with PBS and then incubated for 1.5 hours at room temperature with 50 µl per well of a 1:4000 dilution of horseradish peroxidase-conjugated, affinity purified, goat
35 anti-mouse IgG or IgM heavy and light chain-specific antibodies (HRP; Zymed Laboratories, San Francisco, CA). The wells were then washed three times with PBS, followed by a 30 minute incubation in 50 µl per well of 1 mM 2,2-azino-bis-3-ethylbenzthiazoline-6-sulfonic

-92-

acid (ABTS) in 0.1 M Na-Citrate, pH 4.2 to which a 1:1000 dilution of 30 % hydrogen peroxide had been added as a substrate for HRP to detect bound antibody. The absorbence was then determined at OD₄₁₀ on a spectrophotometric autoreader (Dynatech, Virginia).

Three hybridomas, HA3.1F9, HA5.2B7 and HF2.3D1, were identified that produced 5 antibodies to human B7.2-Ig. HA3.1F9 was determined to be of the IgG1 isotype, HA5.2B7 was determined to be of the IgG2b isotype and HF2.3D1 as determined to be of the IgG2a isotype. Each of these hybridomas were subcloned two additional times to insure that they were monoclonal. Hybridoma cells were deposited with the American Type Culture Collection, which meets the requirements of the Budapest Treaty, on July 19, 1994 as ATCC 10 Accession No. ____ (hybridoma HA3.1F9), ATCC Accession No. ____ (HA5.2B7) and ATCC Accession No. ____ (HF2.3D1).

C. Competitive ELISA

Supernatants from the hybridomas HA3.1F9, HA5.2B7 and HF2.3D1 were further 15 characterized by competitive ELISA, in which the ability of the monoclonal antibodies to inhibit the binding of biotinylated hCTLA4Ig to immobilized hB7-2 immunoglobulin fusion proteins was examined. Biotinylation of hCTLA4Ig was performed using Pierce Immunopure NHS-LC Biotin (Cat. No. 21335). B7-2 immunoglobulin fusion proteins used were: hB7.2-Ig (full-length hB7-2), hB7.2-VIg (hB7-2 variable domain only) and hB7.2-Clg 20 (B7-2 constant domain only). A hB7.1-Ig fusion protein was used as a control. For the ELISA, 96 well plates were coated with the Ig fusion protein (50 µl/well of a 20 µg/ml solution) overnight at room temperature. The wells were washed three times with PBS, blocked with 10 % fetal bovine serum (FBS), 0.1 % bovine serum albumin (BSA) in PBS for 1 hour at room temperature, and washed again three times with PBS. To each well was 25 added 50 µl of Bio-hCTLA4-Ig (70 ng/ml) and 50 µl of competitor monoclonal antibody supernatant. Control antibodies were an anti-B7.1 mAb (EW3.5D12) and the anti-hB7-2 mAb B70 (IgG2b, obtained from Pharmingen). The wells were washed again and streptavidin-conjugated horse radish peroxidase (from Pierce, Cat. No. 21126; 1:2000 dilution, 50 µl/well) was added and incubated for 30 minutes at room temperature. The wells 30 were washed again, followed by a 30 minute incubation in 50 µl per well of ABTS in 0.1 M Na-Citrate, pH 4.2 to which a 1:1000 dilution of 30 % hydrogen peroxide had been added as a substrate for HRP to detect bound antibody. The absorbence was then determined at OD₄₁₀ on a spectrophotometric autoreader (Dynatech, Virginia). The results, shown in Table IV below, demonstrate that each of the mAbs produced by the hybridomas HA3.1F9, 35 HA5.2B7 and HF2.3D1 are able to competitively inhibit the binding of hCTLA4Ig to full-length hB7.2-Ig or hB7.2-VIg (hCTLA4Ig does not bind to hB7.2-Clg).

-93-

TABLE IV

	<u>Blocking of Binding</u>			
	<u>hB7.1-Ig</u>	<u>hB7.2-Ig</u>	<u>hB7.2-Vlg</u>	<u>hB7.2-CIg</u>
EW3.5D12 (anti-hB7.1 mAb)	Yes	No	No	No
B70 (anti-hB7-2)	No	Yes	Yes	No
HA3.1F9 (anti-hB7-2)	No	Yes	Yes	No
HA5.2B7 (anti-hB7-2)	No	Yes	Yes	No
HF2.3D1 (anti-hB7-2)	No	Yes	Yes	No

5 D. Flow Cytometry

Supernatants from the hybridomas HA3.1F9, HA5.2B7 and HF2.3D1 were also characterized by flow cytometry. Supernatants collected from the clones were screened by flow cytometry on CHO and 3T3 cells transfected to express hB7.2 (CHO-hB7.2 and 3T3-hB7.2, respectively) or control transfected 3T3 cells (3T3-Neo). Flow cytometry was performed as follows: 1×10^6 cells were washed three times in 1 % BSA in PBS, then the cells were incubated in 50 μ l hybridoma supernatant or culture media per 1×10^6 cells for 30 minutes at 4 °C. Following the incubation, the cells were washed three times with 1 % BSA in PBS, then incubated in 50 μ l fluorescein-conjugated goat anti-mouse IgG or IgM antibodies (Zymed Laboratories, San Francisco, CA) at 1:50 dilution per 1×10^6 cells for 30 minutes at 4 °C. The cells were then washed three times in 1 % BSA in PBS and fixed with 1 % paraformaldehyde solution. The cell samples were then analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose CA). The results, shown in Figures 17, 18 and 19, demonstrate the monoclonal antibodies produced by the hybridomas HA3.1F9, HA5.2B7 and HF2.3D1 each bind to hB7-2 on the surface of cells.

20

E. Inhibition of Proliferation of Human T Cells by Anti-hB7-2 mAbs

Hybridoma supernatants containing anti-human B7-2 mAbs were tested for their ability to inhibit hB7-2 costimulation of human T cells. In this assay, purified CD28⁺ human T cells were treated with submitogenic amounts of PMA (1ng/ml) to deliver the primary signal and with CHO cells expressing hB7-2 on their surface to deliver the costimulatory signal. Proliferation of the T cells was measured after three days in culture by the addition of ³H-thymidine for the remaining 18 hours. As shown in Table V, resting T cells show little proliferation as measured by ³H-thymidine incorporation (510 pm). Delivery of signal 1 by PMA results in some proliferation (3800 pm) and T cells receiving both the primary (PMA) and costimulatory (CHO/hB7-2) signals proliferate maximally (9020 cpm). All three anti-

-94-

hB7-2 mAbs tested reduce the costimulatory signal induced proliferation to that found for PMA treated cells alone showing that these mAbs can inhibit T cell proliferation by blocking the B7/CD28 costimulatory pathway.

5

TABLE V

Addition to CD28 ⁺ T Cells	hB7-2 mAb	CPM
—	---	510
+PMA	---	3800
+PMA + CHO/hB7-2	---	9020
+PMA + CHO/hB7-2	HF2.301	3030
—	HA5.2B7	1460
—	HA3.1F9	2980

EXAMPLE 9

10 Regression of Implanted Tumor Cells Transfected to Express B7-2

In this example, untransfected or B7-2 transfected J558 plasmacytoma cells were used in tumor regression studies to examine the effect of expression of B7-2 on the surface of tumor cells on the growth of the tumor cells when transplanted into animals.

15 J558 plasmacytoma cells (obtained from the American Type Culture Collection, Rockville, MD; # TIB 6) were transfected with an expression vector containing cDNA encoding either mouse B7-2 (pAWNE03) or B7-1 (pNRDSH or pAWNE03) and a neomycin-resistance gene. Stable transfectants were selected based upon their neomycin resistance and cell surface expression of B7-2 or B7-1 on the tumor cells was confirmed by FACS analysis
20 using either an anti-B7-2 or anti-B7-1 antibody.

25 Syngeneic Balb/c mice, in groups of 5-10 mice/set, were used in experiments designed to determine whether cell-surface expression of B7-2 on tumor cells would result in regression of the implanted tumor cells. Untransfected and transfected J558 cells were cultured *in vitro*, collected, washed and resuspended in Hank's buffered salt solution (GIBCO, Grand Island, New York) at a concentration of 10^8 cells/ml. A patch of skin on the right flank of each mouse was removed of hair with a depilatory and, 24 hours later, 5×10^6 tumor cells/mouse were implanted intradermally or subdermally. Measurements of tumor volume (by linear measurements in three perpendicular directions) were made every two to three days using calipers and a ruler. A typical experiment lasted 18-21 days, after which

-95-

time the tumor size exceeded 10 % of the body mass of mice transplanted with untransfected, control J558 cells. As shown in Figure 20, J558 cells transfected to express B7-2 on their surface were rejected by the mice. No tumor growth was observed even after three weeks. Similar results were observed with J558 cells transfected to express B7-1 on their surface. In
5 contrast, the untransfected (wild-type) J558 cells produced massive tumors in as little as 12 days, requiring the animal to be euthanized. This example demonstrates that cell-surface expression of B7-2 on tumor cells, such as by transfection of the tumor cells with a B7-2 cDNA, induces an anti-tumor response in naive animals that is sufficient to cause rejection of the tumor cells.

10

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

15

-96-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

10

- (A) NAME: DANA-FARBER CANCER INSTITUTE
- (B) STREET: 44 BINNEY STREET
- (C) CITY: BOSTON
- (D) STATE: MASSACHUSETTS
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 02115
- (G) TELEPHONE: (617) 632-4016
- (H) TELEFAX: (617) 632-4012

15

- (A) NAME: REPLIGEN CORPORATION

20

- (B) STREET: ONE KENDALL SQUARE, BLDG 700
- (C) CITY: CAMBRIDGE
- (D) STATE: MASSACHUSETTS
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 02139
- (G) TELEPHONE: (617) 225-6000
- (H) TELEFAX: (617) 494-1975

25

(ii) TITLE OF INVENTION: Novel CTLA4/CD28 Ligands and
Uses Therefor

(iii) NUMBER OF SEQUENCES: 31

30

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: LAHIVE & COCKFIELD
- (B) STREET: 60 State Street, Suite 510
- (C) CITY: Boston
- (D) STATE: Massachusetts
- (E) COUNTRY: USA
- (F) ZIP: 02109

35

(v) COMPUTER READABLE FORM:

40

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

45

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:

50

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US08/101,624; US08/109,393; US08/147,773
- (B) FILING DATE: 26-JUL-1993; 19-AUG-1993; 03-NOV-1993

55

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Mandragouras, Amy E.
- (B) REGISTRATION NUMBER: 36,207
- (C) REFERENCE/DOCKET NUMBER: RPI-004CP2PC

-97-

- (ix) TELECOMMUNICATION INFORMATION:
 (A) TELEPHONE: (617) 227-7400
 (B) TELEFAX: (617) 227-5941

5

(2) INFORMATION FOR SEQ ID NO:1:

- 10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1120 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

- 20 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 107..1093

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACAGGGTGA AAGCTTGCT TCTCTGCTGC TGTAACAGGG ACTAGCACAG ACACACGGAT	60
25 GAGTGGGGTC ATTTCCAGAT ATTAGGTAC AGCAGAACCA GCCAAA ATG GAT CCC	i15
Met Asp Pro	
1	
30 CAG TGC ACT ATG GGA CTG AGT AAC ATT CTC TTT GTG ATG GCC TTC CTG	163
Gln Cys Thr Met Gly Leu Ser Asn Ile Leu Phe Val Met Ala Phe Leu	
5 10 15	
35 CTC TCT GGT GCT CCT CTG AAG ATT CAA GCT TAT TTC AAT GAG ACT	211
Leu Ser Gly Ala Ala Pro Leu Lys Ile Gln Ala Tyr Phe Asn Glu Thr	
20 25 30 35	
40 GCA GAC CTG CCA TGC CAA TTT GCA AAC TCT CAA AAC CAA AGC CTG AGT	259
Ala Asp Leu Pro Cys Gln Phe Ala Asn Ser Gln Asn Gln Ser Leu Ser	
40 45 50	
45 GAG CTA GTA GTA TTT TGG CAG GAC CAG GAA AAC TTG GTT CTG AAT GAG	307
Glu Leu Val Val Phe Trp Gln Asp Gln Glu Asn Leu Val Leu Asn Glu	
55 60 65	
50 GTC TAC TTA GGC AAA GAG AAA TTT GAC AGT GTT CAT TCC AAG TAT ATG	355
Val Tyr Leu Gly Lys Glu Lys Phe Asp Ser Val His Ser Lys Tyr Met	
70 75 80	
50 GGC CGC ACA AGT TTT GAT TCG GAC AGT TGG ACC CTG AGA CTT CAC AAT	403
Gly Arg Thr Ser Phe Asp Ser Asp Ser Trp Thr Leu Arg Leu His Asn	
85 90 95	

-98-

	CTT CAG ATC AAG GAC AAG GGC TTG TAT CAA TGT ATC ATC CAT CAC AAA Leu Gln Ile Lys Asp Lys Gly Leu Tyr Gln Cys Ile Ile His His Lys 100 105 110 115	451
5	AAG CCC ACA GGA ATG ATT CGC ATC CAC CAG ATG AAT TCT GAA CTG TCA Lys Pro Thr Gly Met Ile Arg Ile His Gln Met Asn Ser Glu Leu Ser 120 125 130	499
10	GTG CTT GCT AAC TTC AGT CAA CCT GAA ATA GTA CCA ATT TCT AAT ATA Val Leu Ala Asn Phe Ser Gln Pro Glu Ile Val Pro Ile Ser Asn Ile 135 140 145	547
15	ACA GAA AAT GTG TAC ATA AAT TTG ACC TGC TCA TCT ATA CAC GGT TAC Thr Glu Asn Val Tyr Ile Asn Leu Thr Cys Ser Ser Ile His Gly Tyr 150 155 160	595
20	CCA GAA CCT AAG AAG ATG AGT GTT TTG CTA AGA ACC AAG AAT TCA ACT Pro Glu Pro Lys Lys Met Ser Val Leu Leu Arg Thr Lys Asn Ser Thr 165 170 175	643
25	ATC GAG TAT GAT GGT ATT ATG CAG AAA TCT CAA GAT AAT GTC ACA GAA Ile Glu Tyr Asp Gly Ile Met Gln Lys Ser Gln Asp Asn Val Thr Glu 180 185 190 195	691
30	CTG TAC GAC GTT TCC ATC AGC TTG TCT GTT TCA TTC CCT GAT GTT ACG Leu Tyr Asp Val Ser Ile Ser Leu Ser Val Ser Phe Pro Asp Val Thr 200 205 210	739
35	AGC AAT ATG ACC ATC TTC TGT ATT CTG GAA ACT GAC AAG ACG CGG CTT Ser Asn Met Thr Ile Phe Cys Ile Leu Glu Thr Asp Lys Thr Arg Leu 215 220 225	787
40	TTA TCT TCA CCT TTC TCT ATA GAG CTT GAG GAC CCT CAG CCT CCC CCA Leu Ser Ser Pro Phe Ser Ile Glu Leu Glu Asp Pro Gln Pro Pro Pro 230 235 240	835
45	GAC CAC ATT CCT TGG ATT ACA GCT GTA CTT CCA ACA GTT ATT ATA TGT Asp His Ile Pro Trp Ile Thr Ala Val Leu Pro Thr Val Ile Ile Cys 245 250 255	883
50	GTG ATG GTT TTC TGT CTA ATT CTA TGG AAA TGG AAG AAG AAG AAG CGG Val Met Val Phe Cys Leu Ile Leu Trp Lys Trp Lys Lys Lys Arg 260 265 270 275	931
55	CCT CGC AAC TCT TAT AAA TGT GGA ACC AAC ACA ATG GAG AGG GAA GAG Pro Arg Asn Ser Tyr Lys Cys Gly Thr Asn Thr Met Glu Arg Glu Glu 280 285 290	979
	AGT GAA CAG ACC AAG AAA AGA GAA AAA ATC CAT ATA CCT GAA AGA TCT Ser Glu Gln Thr Lys Lys Arg Glu Lys Ile His Ile Pro Glu Arg Ser 295 300 305	1027
	GAT GAA GCC CAG CGT GTT TTT AAA AGT TCG AAG ACA TCT TCA TGC GAC Asp Glu Ala Gln Arg Val Phe Lys Ser Ser Lys Thr Ser Ser Cys Asp 310 315 320	1075

-99-

AAA AGT GAT ACA TGT TTT TAATTAAAGA GTAAAGCCCA AAAAAAA
Lys Ser Asp Thr Cys Phe
325

1120

5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 329 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp Pro Gln Cys Thr Met Gly Leu Ser Asn Ile Leu Phe Val Met
1 5 10 15

20 Ala Phe Leu Leu Ser Gly Ala Ala Pro Leu Lys Ile Gln Ala Tyr Phe
20 25 30

25 Asn Glu Thr Ala Asp Leu Pro Cys Gln Phe Ala Asn Ser Gln Asn Gln
35 40 45

Ser Leu Ser Glu Leu Val Val Phe Trp Gln Asp Gln Glu Asn Leu Val
50 55 60

30 Leu Asn Glu Val Tyr Leu Gly Lys Glu Lys Phe Asp Ser Val His Ser
65 70 75 80

35 Lys Tyr Met Gly Arg Thr Ser Phe Asp Ser Asp Ser Trp Thr Leu Arg
85 90 95

40 Leu His Asn Leu Gln Ile Lys Asp Lys Gly Leu Tyr Gln Cys Ile Ile
100 105 110

His His Lys Lys Pro Thr Gly Met Ile Arg Ile His Gln Met Asn Ser
115 120 125

Glu Leu Ser Val Leu Ala Asn Phe Ser Gln Pro Glu Ile Val Pro Ile
130 135 140

45 Ser Asn Ile Thr Glu Asn Val Tyr Ile Asn Leu Thr Cys Ser Ser Ile
145 150 155 160

His Gly Tyr Pro Glu Pro Lys Lys Met Ser Val Leu Leu Arg Thr Lys
165 170 175

50 Asn Ser Thr Ile Glu Tyr Asp Gly Ile Met Gln Lys Ser Gln Asp Asn
180 185 190

Val Thr Glu Leu Tyr Asp Val Ser Ile Ser Leu Ser Val Ser Phe Pro
55 195 200 205

-100-

Asp Val Thr Ser Asn Met Thr Ile Phe Cys Ile Leu Glu Thr Asp Lys
210 215 220

5 Thr Arg Leu Leu Ser Ser Pro Phe Ser Ile Glu Leu Glu Asp Pro Gln
225 230 235 240

Pro Pro Pro Asp His Ile Pro Trp Ile Thr Ala Val Leu Pro Thr Val
245 250 255

10 Ile Ile Cys Val Met Val Phe Cys Leu Ile Leu Trp Lys Trp Lys Lys
260 265 270

Lys Lys Arg Pro Arg Asn Ser Tyr Lys Cys Gly Thr Asn Thr Met Glu
15 275 280 285

Arg Glu Glu Ser Glu Gln Thr Lys Lys Arg Glu Lys Ile His Ile Pro
290 295 300

20 Glu Arg Ser Asp Glu Ala Gln Arg Val Phe Lys Ser Ser Lys Thr Ser
305 310 315 320

Ser Cys Asp Lys Ser Asp Thr Cys Phe
325

25 (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
30 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

40 TAATACGACT CACTATAGGG 20

(2) INFORMATION FOR SEQ ID NO:4:

- 45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

55

-101-

TAAGGTTCT TCACAAAG

18

(2) INFORMATION FOR SEQ ID NO:5:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: oligonucleotide

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACTGGTAGGT ATGGAAGATC C

21

(2) INFORMATION FOR SEQ ID NO:6:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: oligonucleotide

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGCGAATCA TTCCCTGTGGG C

21

(2) INFORMATION FOR SEQ ID NO:7:

- 35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: oligonucleotide

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAAGCCCACA GGAATGATTG G

21

(2) INFORMATION FOR SEQ ID NO:8:

- 50 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid

-102-

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

10 CTCTCAAAAC CAAAGCCTGA G

21

(2) INFORMATION FOR SEQ ID NO:9:

- 15 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: oligonucleotide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTAGGTCACA GCAGAACGAG C

21

(2) INFORMATION FOR SEQ ID NO:10:

- 30 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: oligonucleotide

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCTGGAACT GACAAGACGC G

21

(2) INFORMATION FOR SEQ ID NO:11:

- 45 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: oligonucleotide

55

-103-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTCAGGCTTT GGTTTGAGA G

21

5 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CACTCTCTTC CCTCTCCATT G

21

20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

35 GACAAGCTGA TGGAAACGTC G

21

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

40

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: oligonucleotide

50

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CAATGGAGAG GGAAGAGAGT G

21

(2) INFORMATION FOR SEQ ID NO:15:

55

(i) SEQUENCE CHARACTERISTICS:

-104-

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: oligonucleotide

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTTTAGAGCA CA

12

15 (2) INFORMATION FOR SEQ ID NO:16:
15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCTAAAG

8

30 (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: peptide

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Lys Tyr Met Gly Arg Thr Ser Phe Asp
5

45

(2) INFORMATION FOR SEQ ID NO:18:

50

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-105-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

5 Lys Ser Gln Asp Asn Val Thr Glu Lys Tyr Asp Val Ser
5 10

(2) INFORMATION FOR SEQ ID NO:19:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

20 Trp Lys Trp Lys Lys Lys Arg Pro Arg Asn Ser Tyr Lys Cys
5 10 15

(2) INFORMATION FOR SEQ ID NO:20:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
TGGCCCATGG CTTCAGA

17

40 (2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
45 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
GCCAAATGG ATCCCCA

17

-106-

(2) INFORMATION FOR SEQ ID NO:22:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1163 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
15 (A) NAME/KEY: CDS
 (B) LOCATION: 111..1040

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

20	CCCACGCGTC CGGGAGCAAG CAGACGCGTA AGAGTGGCTC CTGTAGGCAG CACGGACTTG AAACAACCAGA CTCCCTGTAGA CGTGTCCAG AACTTACGGA AGCACCCACG ATG GAC Met Asp	60
25	1 CCC AGA TGC ACC ATG GGC TTG GCA ATC CTT ATC TTT GTG ACA GTC TTG Pro Arg Cys Thr Met Gly Leu Ala Ile Leu Ile Phe Val Thr Val Leu 5 10 15	164
30	CTG ATC TCA GAT GCT GTT TCC GTG GAG ACG CAA GCT TAT TTC AAT GGG Leu Ile Ser Asp Ala Val Ser Val Glu Thr Gln Ala Tyr Phe Asn Gly 20 25 30	212
35	ACT GCA TAT CTG CCG TGC CCA TTT ACA AAG GCT CAA AAC ATA AGC CTG Thr Ala Tyr Leu Pro Cys Pro Phe Thr Lys Ala Gln Asn Ile Ser Leu 35 40 45 50	260
40	AGT GAG CTG GTA GTA TTT TGG CAG GAC CAG CAA AAG TTG GTT CTG TAC Ser Glu Leu Val Val Phe Trp Gln Asp Gln Gln Lys Leu Val Leu Tyr 55 60 65	308
45	GAG CAC TAT TTG GGC ACA GAG AAA CTT GAT AGT GTG AAT GCC AAG TAC Glu His Tyr Leu Gly Thr Glu Lys Leu Asp Ser Val Asn Ala Lys Tyr 70 75 80	356
50	CTG GGC CGC ACG AGC TTT GAC AGG AAC AAC TGG ACT CTA CGA CTT CAC Leu Gly Arg Thr Ser Phe Asp Arg Asn Asn Trp Thr Leu Arg Leu His 85 90 95	404
55	AAT GTT CAG ATC AAG GAC ATG GGC TCG TAT GAT TGT TTT ATA CAA AAA Asn Val Gln Ile Lys Asp Met Gly Ser Tyr Asp Cys Phe Ile Gln Lys 100 105 110	452

-107-

	AAG CCA CCC ACA GGA TCA ATT ATC CTC CAA CAG ACA TTA ACA GAA CTG Lys Pro Pro Thr Gly Ser Ile Ile Leu Gln Gln Thr Leu Thr Glu Leu 115 120 125 130	500
5	TCA GTG ATC GCC AAC TTC AGT GAA CCT GAA ATA AAA CTG GCT CAG AAT Ser Val Ile Ala Asn Phe Ser Glu Pro Glu Ile Lys Leu Ala Gln Asn 135 140 145	548
10	GTA ACA GGA AAT TCT GGC ATA AAT TTG ACC TGC ACG TCT AAG CAA GGT Val Thr Gly Asn Ser Gly Ile Asn Leu Thr Cys Thr Ser Lys Gin Gly 150 155 160	596
15	CAC CCG AAA CCT AAG AAG ATG TAT TTT CTG ATA ACT AAT TCA ACT AAT His Pro Lys Pro Lys Lys Met Tyr Phe Leu Ile Thr Asn Ser Thr Asn 165 170 175	644
20	GAG TAT GGT GAT AAC ATG CAG ATA TCA CAA GAT AAT GTC ACA GAA CTG Glu Tyr Gly Asp Asn Met Gln Ile Ser Gln Asp Asn Val Thr Glu Leu 180 185 190	692
25	TTC AGT ATC TCC AAC AGC CTC TCT CTT TCA TTC CCG GAT GGT GTG TGG Phe Ser Ile Ser Asn Ser Leu Ser Leu Ser Phe Pro Asp Gly Val Trp 195 200 205 210	740
30	CAT ATG ACC GTT GTG TGT GTT CTG GAA ACG GAG TCA ATG AAG ATT TCC His Met Thr Val Val Cys Val Leu Glu Thr Glu Ser Met Lys Ile Ser 215 220 225	788
35	TCC AAA CCT CTC AAT TTC ACT CAA GAG TTT CCA TCT CCT CAA ACG TAT Ser Lys Pro Leu Asn Phe Thr Gln Glu Phe Pro Ser Pro Gln Thr Tyr 230 235 240	836
40	TGG AAG GAG ATT ACA GCT TCA GTT ACT GTG GCC CTC CTC CTT GTG ATG Trp Lys Glu Ile Thr Ala Ser Val Thr Val Ala Leu Leu Val Met 245 250 255	884
45	CTG CTC ATC ATT GTA TGT CAC AAG AAG CCG AAT CAG CCT AGC AGG CCC Leu Leu Ile Ile Val Cys His Lys Lys Pro Asn Gln Pro Ser Arg Pro 260 265 270	932
50	AGC AAC ACA GCC TCT AAG TTA GAG CGG GAT AGT AAC GCT GAC AGA GAG Ser Asn Thr Ala Ser Lys Leu Glu Arg Asp Ser Asn Ala Asp Arg Glu 275 280 285 290	980
55	ACT ATC AAC CTG AAG GAA CTT GAA CCC CAA ATT GCT TCA GCA AAA CCA Thr Ile Asn Leu Lys Glu Leu Glu Pro Gln Ile Ala Ser Ala Lys Pro 295 300 305	1028
	AAT GCA GAG TGAAGGCAGT GAGAGCCTGA GGAAAGAGTT AAAAATTGCT Asn Ala Glu	1077
	TTGCCTGAAA TAAGAAGTGC AGAGTTCTC AGAATTCAA AATGTTCTCA GCTGATTGGA	1137
	ATTCTACAGT TGAATAATTA AAGAAC	1163

-108-

(2) INFORMATION FOR SEQ ID NO:23:

5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 309 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

15 Met Asp Pro Arg Cys Thr Met Gly Leu Ala Ile Leu Ile Phe Val Thr
 1 5 10 15

20 Val Leu Leu Ile Ser Asp Ala Val Ser Val Glu Thr Gln Ala Tyr Phe
 20 25 30

25 Asn Gly Thr Ala Tyr Leu Pro Cys Pro Phe Thr Lys Ala Gln Asn Ile
 35 40 45

30 Ser Leu Ser Glu Leu Val Val Phe Trp Gln Asp Gln Gln Lys Leu Val
 50 55 60

35 Leu Tyr Glu His Tyr Leu Gly Thr Glu Lys Leu Asp Ser Val Asn Ala
 65 70 75 80

40 Lys Tyr Leu Gly Arg Thr Ser Phe Asp Arg Asn Asn Trp Thr Leu Arg
 85 90 95

45 Leu His Asn Val Gln Ile Lys Asp Met Gly Ser Tyr Asp Cys Phe Ile
 100 105 110

50 Gln Lys Lys Pro Pro Thr Gly Ser Ile Ile Leu Gln Gln Thr Leu Thr
 115 120 125

55 Glu Leu Ser Val Ile Ala Asn Phe Ser Gly Pro Glu Ile Lys Leu Ala
 130 135 140

60 Gln Asn Val Thr Gly Asn Ser Gly Ile Asn Leu Thr Cys Thr Ser Lys
 145 150 155 160

65 Gln Gly His Pro Lys Pro Lys Lys Met Tyr Phe Leu Ile Thr Asn Ser
 165 170 175

70 Thr Asn Glu Tyr Gly Asp Asn Met Gln Ile Ser Gln Asp Asn Val Thr
 180 185 190

75 Glu Leu Phe Ser Ile Ser Asn Ser Leu Ser Leu Ser Phe Pro Asp Gly
 195 200 205

80 Val Trp His Met Thr Val Val Cys Val Leu Glu Thr Glu Ser Met Lys
 210 215 220

-109-

Ile Ser Ser Lys Pro Leu Asn Phe Thr Gln Glu Phe Pro Ser Pro Gln
225 230 235 240

Thr Tyr Trp Lys Glu Ile Thr Ala Ser Val Thr Val Ala Leu Leu Leu
5 245 250 255

Val Met Leu Leu Ile Ile Val Cys His Lys Lys Pro Asn Gln Pro Ser
260 265 270

10 Arg Pro Ser Asn Thr Ala Ser Lys Leu Glu Arg Asp Ser Asn Ala Asp
275 280 285

Arg Glu Thr Ile Asn Leu Lys Glu Leu Glu Pro Gln Ile Ala Ser Ala
290 295 300

15 Lys Pro Asn Ala Glu
305

20 (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: oligonucleotide

30
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:
ACATAAGCCT GAGTGAGCTG G

21

35

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: oligonucleotide

50
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
ATGATGAGCA GCATCACAAAG G

21

(2) INFORMATION FOR SEQ ID NO:26:

-110-

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: oligonucleotide

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TGGTCGAGTG AGTCCGAATA C

21

15 (2) INFORMATION FOR SEQ ID NO:27:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: oligonucleotide

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GACGAGTAGT AACATACAGT G

21

35 (2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 1491 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

50 (vi) ORIGINAL SOURCE:

- 55 (A) ORGANISM: Homo sapien
(F) TISSUE TYPE: lymphoid
(G) CELL TYPE: B cell
(H) CELL LINE: Raji

-111-

(vii) IMMEDIATE SOURCE:

- 5 (A) LIBRARY: cDNA in pCDM8 vector
 (B) CLONE: B7, Raji clone #13

(viii) POSITION IN GENOME:

- 10 (A) CHROMOSOME SEGMENT: 3

15 (ix) FEATURE:

- (A) NAME/KEY: Open reading frame (translated region)
 (B) LOCATION: 318 to 1181 bp
 (C) IDENTIFICATION METHOD: similarity to other pattern

20 (ix) FEATURE:

- (A) NAME/KEY: Alternate polyadenylation signal
 (B) LOCATION: 1474 to 1479 bp
 (C) IDENTIFICATION METHOD: similarity to other pattern

25 (x) PUBLICATION INFORMATION:

- (A) AUTHORS: FREEMAN, GORDON J.
 FREEDMAN, ARNOLD S.
 SEGIL, JEFFREY M.
 LEE, GRACE
 WHITMAN, JAMES F.
 NADLER, LEE M.

- 30 (B) TITLE: B7, A New Member Of The Ig Superfamily With
 Unique Expression On Activated And Neoplastic B Cells

- 35 (C) JOURNAL: The Journal of Immunology
 (D) VOLUME: 143
 (E) ISSUE: 8
 (F) PAGES: 2714-2722
 (G) DATE: 15-OCT-1989
 (H) RELEVANT RESIDUES In SEQ ID NO:28: FROM 1 TO 1491

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

45 CCAAAGAAAA AGTGATTTGT CATTGTTTA TAGACTGTAA GAAGAGAACCA TCTCAGAAGT 60
 GGAGTCTTAC CCTGAAATCA AAGGATTTAA AGAAAAAGTG GAATTTTCT TCAGCAAGCT 120
 GTGAAACTAA ATCCACAAACC TTTGGAGACC CAGGAACACC CTCCAATCTC TGTGTGTTTT 180
50 GTAAACATCA CTGGAGGGTC TTCTACGTGA GCAAATGGAT TGTCATCAGC CCTGCCTGTT 240
 TTGCACCTGG GAAGTGCCCT GGTCTTACTT GGGTCCAAAT TGTTGGCTTT CACTTTGAC 300

-112-

CCTAAGCATC TGAAGGCC ATG GGC CAC ACA CGG AGG CAG GGA ACA TCA CCA TCC 353
 Met Gly His Thr Arg Arg Gln Gly Thr Ser Pro Ser
 -30 -25

5

AAG TGT CCA TAC CTG AAT TTC TTT CAG CTC TTG GTG CTG GCT GGT CTT 401
 Lys Cys Pro Tyr Leu Asn Phe Phe Gln Leu Leu Val Leu Ala Gly Leu
 -20 -15 -10

10

TCT CAC TTC TGT TCA GGT GTT ATC CAC GTG ACC AAG GAA GTG AAA GAA 449
 Ser His Phe Cys Ser Gly Val Ile His Val Thr Lys Glu Val Lys Glu
 -5 1 5 10

15

GTG GCA ACG CTG TCC TGT GGT CAC AAT GTT TCT GTT GAA GAG CTG GCA 497
 Val Ala Thr Leu Ser Cys Gly His Asn Val Ser Val Glu Glu Leu Ala
 15 20 25

20

CAA ACT CGC ATC TAC TGG CAA AAG GAG AAG AAA ATG GTG CTG ACT ATG 545
 Gln Thr Arg Ile Tyr Trp Gln Lys Glu Lys Lys Met Val Leu Thr Met
 30 35 40

25

ATG TCT GGG GAC ATG AAT ATA TGG CCC GAG TAC AAG AAC CGG ACC ATC 593
 Met Ser Gly Asp Met Asn Ile Trp Pro Glu Tyr Lys Asn Arg Thr Ile
 45 50 55

30

TTT GAT ATC ACT AAT AAC CTC TCC ATT GTG ATC CTG GCT CTG CGC CCA 641
 Phe Asp Ile Thr Asn Asn Leu Ser Ile Val Ile Leu Ala Leu Arg Pro
 60 65 70

35

TCT GAC GAG GGC ACA TAC GAG TGT GTT CTG AAG TAT GAA AAA GAC 689
 Ser Asp Glu Gly Thr Tyr Glu Cys Val Val Leu Lys Tyr Glu Lys Asp
 75 80 85 90

40

GCT TTC AAG CGG GAA CAC CTG GCT GAA GTG ACG TTA TCA GTC AAA GCT 737
 Ala Phe Lys Arg Glu His Leu Ala Glu Val Thr Leu Ser Val Lys Ala
 95 100 105

45

GAC TTC CCT ACA CCT AGT ATA TCT GAC TTT GAA ATT CCA ACT TCT AAT 785
 Asp Phe Pro Thr Pro Ser Ile Ser Asp Phe Glu Ile Pro Thr Ser Asn
 110 115 120

50

ATT AGA AGG ATA ATT TGC TCA ACC TCT GGA GGT TTT CCA GAG CCT CAC 833
 Ile Arg Arg Ile Ile Cys Ser Thr Ser Gly Gly Phe Pro Glu Pro His
 125 130 135

55

-113-

CTC TCC TGG TTG GAA AAT GGA GAA GAA TTA AAT GCC ATC AAC ACA ACA 881
 Leu Ser Trp Leu Glu Asn Gly Glu Glu Leu Asn Ala Ile Asn Thr Thr
 140 145 150

5 GTT TCC CAA GAT CCT GAA ACT GAG CTC TAT GCT GTT AGC AGC AAA CTG 929
 Val Ser Gln Asp Pro Glu Thr Glu Leu Tyr Ala Val Ser Ser Lys Leu
 155 160 165 170

10 GAT TTC AAT ATG ACA ACC AAC CAC AGC TTC ATG TGT CTC ATC AAG TAT 977
 Asp Phe Asn Met Thr Thr Asn His Ser Phe Met Cys Leu Ile Lys Tyr
 175 180 185

15 GGA CAT TTA AGA GTG AAT CAG ACC TTC AAC TGG AAT ACA ACC AAG CAA 1025
 Gly His Leu Arg Val Asn Gln Thr Phe Asn Trp Asn Thr Thr Lys Gln
 190 195 200

20 GAG CAT TTT CCT GAT AAC CTG CTC CCA TCC TGG GCC ATT ACC TTA ATC 1073
 Glu His Phe Pro Asp Asn Leu Leu Pro Ser Trp Ala Ile Thr Leu Ile
 205 210 215

25 TCA GTA AAT GGA ATT TTT GTG ATA TGC TGC CTG ACC TAC TGC TTT GCC 1121
 Ser Val Asn Gly Ile Phe Val Ile Cys Cys Leu Thr Tyr Cys Phe Ala
 220 225 230

30 CCA AGA TGC AGA GAG AGA AGG AGG AAT GAG AGA TTG AGA AGG GAA AGT 1169
 Pro Arg Cys Arg Glu Arg Arg Asn Glu Arg Leu Arg Arg Glu Ser
 235 240 245 250

35 GTA CGC CCT GTA TAACAGTGTC CGCAGAAGCA AGGGGCTGAA AAGATCTGAA 1221
 Val Arg Pro Val

40 GGTAGCCTCC GTCATCTCTT CTGGGATACA TGGATCGTGG GGATCATGAG GCATTCTTCC 1281

CTTAACAAAT TTAAGCTGTT TTACCCACTA CCTCACCTTC TTAAAAACCT CTTTCAGATT 1341

45 AAGCTGAACA GTTACAAGAT GGCTGGCATC CCTCTCCTTT CTCCCCATAT GCAATTTGCT 1401

TAATGTAACC TCTTCTTTG CCATGTTGCC ATTCTGCCAT CTTGAATTGT CTTGTCAGCC 1461

50 AATTCAATTAT CTATTAACCA CTAATTTGAG 1491

-114-

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 288 amino acids
 (B) TYPE: amino acid
 (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 10 (A) DESCRIPTION: B cell activation antigen; natural ligand
 for CD28 T cell surface antigen; transmembrane protein

(ix) FEATURE:

- 15 (A) NAME/KEY: signal sequence
 (B) LOCATION: -34 to -1
 (C) IDENTIFICATION METHOD: amino terminal sequencing of
 soluble protein
 (D) OTHER INFORMATION: hydrophobic

20 (ix) FEATURE:

- 25 (A) NAME/KEY: extracellular domain
 (B) LOCATION: 1 to 208
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

30 (ix) FEATURE:

- 35 (A) NAME/KEY: transmembrane domain
 (B) LOCATION: 209 to 235
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

40 (ix) FEATURE:

- 45 (A) NAME/KEY: intracellular domain
 (B) LOCATION: 236 to 254
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

50 (ix) FEATURE:

- 55 (A) NAME/KEY: N-linked glycosylation
 (B) LOCATION: 19 to 21
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

(ix) FEATURE:

- 55 (A) NAME/KEY: N-linked glycosylation

-115-

- (B) LOCATION: 55 to 57
(C) IDENTIFICATION METHOD: similarity with known sequence

5

(ix) FEATURE:

- (A) NAME/KEY: N-linked glycosylation
(B) LOCATION: 64 to 66
10 (C) IDENTIFICATION METHOD: similarity with known sequence

(ix) FEATURE:

15

- (A) NAME/KEY: N-linked glycosylation
(B) LOCATION: 152 to 154
10 (C) IDENTIFICATION METHOD: similarity with known sequence

20

(ix) FEATURE:

25

- (A) NAME/KEY: N-linked glycosylation
(B) LOCATION: 173 to 175
25 (C) IDENTIFICATION METHOD: similarity with known sequence

30

(ix) FEATURE:

35

- (A) NAME/KEY: N-linked glycosylation
(B) LOCATION: 177 to 179
35 (C) IDENTIFICATION METHOD: similarity with known sequence

(ix) FEATURE:

40

- (A) NAME/KEY: N-linked glycosylation
(B) LOCATION: 192 to 194
40 (C) IDENTIFICATION METHOD: similarity with known sequence

45

(ix) FEATURE:

50

- (A) NAME/KEY: N-linked glycosylation
(B) LOCATION: 198 to 200
50 (C) IDENTIFICATION METHOD: similarity with known sequence

-116-

(ix) FEATURE:

- 5 (A) NAME/KEY: Ig V-set domain
 (B) LOCATION: 1 to 104
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

10 (ix) FEATURE:
10 (A) NAME/KEY: Ig C-set domain
 (B) LOCATION: 105 to 202
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

15 (x) PUBLICATION INFORMATION:
15 (A) AUTHORS: FREEMAN, GORDON J.
 FREEDMAN, ARNOLD S.
20 SEGIL, JEFFREY M.
 LEE, GRACE
 WHITMAN, JAMES F.
 NADLER, LEE M.

- 25 (B) TITLE: B7, A New Member Of The Ig Superfamily With
 Unique Expression On Activated And Neoplastic B Cells
 (C) JOURNAL: The Journal of Immunology
 (D) VOLUME: 143
 (E) ISSUE: 8
30 (F) PAGES: 2714-2722
 (G) DATE: 15-OCT-1989
 (H) RELEVANT RESIDUES IN SEQUENCE ID NO:29: From -26 to 262

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:
35

Met Gly His Thr Arg Arg Gln Gly Thr Ser Pro Ser Lys Cys Pro Tyr
40 -30 -25 -20
Leu Asn Phe Phe Gln Leu Leu Val Leu Ala Gly Leu Ser His Phe Cys
45 -15 -10 -5
Ser Gly Val Ile His Val Thr Lys Glu Val Lys Glu Val Ala Thr Leu
45 -1 1 5 10
Ser Cys Gly His Asn Val Ser Val Glu Glu Leu Ala Gln Thr Arg Ile
50 15 20 25 30
Tyr Trp Gln Lys Glu Lys Met Val Leu Thr Met Met Ser Gly Asp
50 35 40 45
Met Asn Ile Trp Pro Glu Tyr Lys Asn Arg Thr Ile Phe Asp Ile Thr
55 50 55 60
55

-117-

	Asn Asn Leu Ser Ile Val Ile Leu Ala Leu Arg Pro Ser Asp Glu Gly			
	65	70	75	
	Thr Tyr Glu Cys Val Val Leu Lys Tyr Glu Lys Asp Ala Phe Lys Arg			
5	80	85	90	
	Glu His Leu Ala Glu Val Thr Leu Ser Val Lys Ala Asp Phe Pro Thr			
	95	100	105	110
10	Pro Ser Ile Ser Asp Phe Glu Ile Pro Thr Ser Asn Ile Arg Arg Ile			
	115	120	125	
	Ile Cys Ser Thr Ser Gly Gly Phe Pro Glu Pro His Leu Ser Trp Leu			
	130	135	140	
15	Glu Asn Gly Glu Glu Leu Asn Ala Ile Asn Thr Thr Val Ser Gln Asp			
	145	150	155	
20	Pro Glu Thr Glu Leu Tyr Ala Val Ser Ser Lys Leu Asp Phe Asn Met			
	160	165	170	
	Thr Thr Asn His Ser Phe Met Cys Leu Ile Lys Tyr Gly His Leu Arg			
	175	180	185	190
25	Val Asn Gln Thr Phe Asn Trp Asn Thr Thr Lys Gln Glu His Phe Pro			
	195	200	205	
30	Asp Asn Leu Leu Pro Ser Trp Ala Ile Thr Leu Ile Ser Val Asn Gly			
	210	215	220	
	Ile Phe Val Ile Cys Cys Leu Thr Tyr Cys Phe Ala Pro Arg Cys Arg			
	225	230	235	
35	Glu Arg Arg Arg Asn Glu Arg Leu Arg Arg Glu Ser Val Arg Pro Val			
	240	245	250	
40	(4) INFORMATION FOR SEQ ID NO:30:			
	(i) SEQUENCE CHARACTERISTICS:			
45	(A) LENGTH: 1716 base pairs			
	(B) TYPE: nucleic acid			
	(C) STRANDEDNESS: double			
	(D) TOPOLOGY: linear			
50	(ii) MOLECULAR TYPE: cDNA to mRNA			
	(iii) HYPOTHETICAL: no			
	(vi) ORIGINAL SOURCE:			
55	(A) ORGANISM: <u>Mus musculus</u>			

-119-

	TCT GTC ATT GCT GGG AAA CTA AAA GTG TGG CCC GAG TAT AAG AAC CGG	530
	Ser Val Ile Ala Gly Lys Leu Lys Val Trp Pro Glu Tyr Lys Asn Arg	
	45	50
	55	
5	ACT TTA TAT GAC AAC ACT ACC TAC TCT CTT ATC ATC CTG GGC CTG GTC	578
	Thr Leu Tyr Asp Asn Thr Thr Tyr Ser Leu Ile Ile Leu Gly Leu Val	
	60	65
	70	
10	CTT TCA GAC CGG GGC ACA TAC AGC TGT GTC GTT CAA AAG AAG GAA AGA	626
	Leu Ser Asp Arg Gly Thr Tyr Ser Cys Val Val Gln Lys Lys Glu Arg	
	75	80
	85	
15	GGA ACG TAT GAA GTT AAA CAC TTG GCT TTA GTA AAG TTG TCC ATC AAA	674
	Gly Thr Tyr Glu Val Lys His Leu Ala Leu Val Lys Leu Ser Ile Lys	
	90	95
	100	105
20	GCT GAC TTC TCT ACC CCC AAC ATA ACT GAG TCT GGA AAC CCA TCT GCA	722
	Ala Asp Phe Ser Thr Pro Asn Ile Thr Glu Ser Gly Asn Pro Ser Ala	
	110	115
	120	
	GAC ACT AAA AGG ATT ACC TGC TTT GCT TCC GGG GGT TTC CCA AAG CCT	770
	Asp Thr Lys Arg Ile Thr Cys Phe Ala Ser Gly Phe Pro Lys Pro	
	125	130
	135	
25	CGC TTC TCT TGG TTG GAA AAT GGA AGA GAA TTA CCT GGC ATC AAT ACG	818
	Arg Phe Ser Trp Leu Glu Asn Gly Arg Glu Leu Pro Gly Ile Asn Thr	
	140	145
	150	
30	ACA ATT TCC CAG GAT CCT GAA TCT GAA TTG TAC ACC ATT AGT AGC CAA	866
	Thr Ile Ser Gln Asp Pro Glu Ser Glu Leu Tyr Thr Ile Ser Ser Gln	
	155	160
	165	
35	CTA GAT TTC AAT ACG ACT CGC AAC CAC ACC ATT AAG TGT CTC ATT AAA	914
	Leu Asp Phe Asn Thr Thr Arg Asn His Thr Ile Lys Cys Leu Ile Lys	
	170	175
	180	185
40	TAT GGA GAT GCT CAC GTG TCA GAG GAC TTC ACC TGG GAA AAA CCC CCA	962
	Tyr Gly Asp Ala His Val Ser Glu Asp Phe Thr Trp Glu Lys Pro Pro	
	190	195
	200	
	GAA GAC CCT CCT GAT AGC AAG AAC ACA CTT GTG CTC TTT GGG GCA GGA	1010
	Glu Asp Pro Pro Asp Ser Lys Asn Thr Leu Val Leu Phe Gly Ala Gly	
	205	210
	215	
45	TTC GGC GCA GTA ATA ACA GTC GTC GTC ATC GTT GTC ATC ATC AAA TGC	1058
	Phe Gly Ala Val Ile Thr Val Val Val Ile Val Val Ile Ile Lys Cys	
	220	225
	230	
50	TTC TGT AAG CAC AGA AGC TGT TTC AGA AGA AAT GAG GCA AGC AGA GAA	1106
	Phe Cys Lys His Arg Ser Cys Phe Arg Arg Asn Glu Ala Ser Arg Glu	
	235	240
	245	
55	ACA AAC AAC AGC CTT ACC TTC GGG CCT GAA GAA GCA TTA GCT GAA CAG	1154
	Thr Asn Asn Ser Leu Thr Phe Gly Pro Glu Glu Ala Leu Ala Glu Gln	
	250	255
	260	265

-120-

	ACC GTC TTC CTT TAGTTCTCT CTGTCCATGT GGGATACATG GTATTATGTG	1206
	Thr Val Phe Leu	
5	GCTCATGAGG TACAATCTT CTTTCAGCAC CGTGCTAGCT GATCTTCGG ACAACTTGAC	1266
	ACAAGATAGA GTTAACTGGG AAGAGAAAGC CTTGAATGAG GATTCTTTC CATCAGGAAG	1326
	CTACGGGCAA GTTTGCTGGG CCTTTGATIG CTTGATGACT GAAGTGGAAA GGCTGAGCCC	1386
10	ACTGTGGGTG GTGCTAGCCC TGGCAGGGG CAGGTGACCC TGGGTGGTAT AAGAAAAAGA	1446
	GCTGTCACTA AAAGGAGAGG TGCCTAGTCT TACTGCAACT TGATATGTCA TGTTTGGTG	1506
15	GTGTCTGTGG GAGGCCTGCC CTTTCTGAA GAGAAGTGGT GGGAGAGTGG ATGGGTGGG	1566
	GGCAGAGGAA AAGTGGGGA GAGGGCCTGG GAGGAGAGGA GGGAGGGGA CGGGGTGGG	1626
	GTGGGGAAAA CTATGGTTGG GATGTAAAAA CGGATAATAA TATAAATATT AAATAAAAAG	1686
20	AGAGTATTGA GCAAAAAAAA AAAAAAAA	1716

(5) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 306 amino acids
- (B) TYPE: amino acid
- 30 (C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 35 (A) DESCRIPTION: B lymphocyte activation antigen; Ig superfamily member; T cell costimulatory signal via activation of CD28 pathways, binds to CD28⁺ T cells, transmembrane protein

(ix) FEATURE:

- 40 (A) NAME/KEY: signal sequence
- (B) LOCATION: -37 to -1
- (C) IDENTIFICATION METHOD: similarity with known sequence
- 45 (D) OTHER INFORMATION: hydrophobic

(ix) FEATURE:

- 50 (A) NAME/KEY: extracellular domain
- (B) LOCATION: 1 to 210
- (C) IDENTIFICATION METHOD: similarity with known sequence

-121-

(ix) FEATURE:

- 5 (A) NAME/KEY: transmembrane domain
 (B) LOCATION: 211 to 235
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

(ix) FEATURE:

- 10 (A) NAME/KEY: intracellular (cytoplasmic) domain
 (B) LOCATION: 236 to 269
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

15

(ix) FEATURE:

- 20 (A) NAME/KEY: Ig V-set domain
 (B) LOCATION: 1 to 105
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

25

(ix) FEATURE:

- 25 (A) NAME/KEY: Ig C-set domain
 (B) LOCATION: 106 to 199
 (C) IDENTIFICATION METHOD: similarity with known
 sequence

30

(x) PUBLICATION INFORMATION:

- 35 (A) AUTHORS: FREEMAN, GORDON J.
 GRAY, GARY S.
 GIMMI, CLAUDE D.
 LOMBARD, DAVID B.
 ZHOU, LIANG-JI
 WHITE, MICHAEL
40 (FINGEROTH, JOYCE D.
 GRIBBEN, JOHN G.
 NADLER, LEE M.
- 45 (B) TITLE: Structure, Expression, and T Cell Costimulatory
 Activity Of The Murine Homologue Of The Human B
 Lymphocyte Activation Antigen B7
 (C) JOURNAL: Journal of Experimental Medicine
 (D) VOLUME:
 (E) ISSUE:
50 (F) PAGES:
 (G) DATE: IN PRESS
 (H) RELEVANT RESIDUES IN SEQUENCE ID NO:31: From -37 to 269

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

-122-

Met Ala Cys Asn Cys Gln Leu Met Gln Asp Thr Pro Leu Leu Lys Phe
-35 -30 -25

5 Pro Cys Pro Arg Leu Ile Leu Leu Phe Val Leu Leu Ile Arg Leu Ser
-20 -15 -10

10 Gln Val Ser Ser Asp Val Asp Glu Gln Leu Ser Lys Ser Val Lys Asp
-5 -1 1 5 10

Lys Val Leu Leu Pro Cys Arg Tyr Asn Ser Pro His Glu Asp Glu Ser
15 20 25

15 Glu Asp Arg Ile Tyr Trp Gln Lys His Asp Lys Val Val Leu Ser Val
30 35 40

Ile Ala Gly Lys Leu Lys Val Trp Pro Glu Tyr Lys Asn Arg Thr Leu
20 45 50 55

Tyr Asp Asn Thr Thr Tyr Ser Leu Ile Ile Leu Gly Leu Val Leu Ser
60 65 70 75

25 Asp Arg Gly Thr Tyr Ser Cys Val Val Gln Lys Lys Glu Arg Gly Thr
80 85 90

Tyr Gly Val Lys His Leu Ala Leu Val Lys Leu Ser Ile Lys Ala Asp
95 100 105

30 Phe Ser Thr Pro Asn Ile Thr Glu Ser Gly Asn Pro Ser Ala Asp Thr
110 115 120

Lys Arg Ile Thr Cys Phe Ala Ser Gly Gly Phe Pro Lys Pro Arg Phe
35 125 130 135

Ser Trp Leu Glu Asn Gly Arg Glu Leu Pro Gly Ile Asn Thr Thr Ile
140 145 150 155

40 Ser Gln Asp Pro Glu Ser Glu Leu Tyr Thr Ile Ser Ser Gln Leu Asp
160 165 170

Phe Asn Thr Thr Arg Asn His Thr Ile Lys Cys Leu Ile Lys Tyr Gly
175 180 185

45 Asp Ala His Val Ser Glu Asp Phe Thr Trp Glu Lys Pro Pro Glu Asp
190 195 200

Pro Pro Asp Ser Lys Asn Thr Leu Val Leu Phe Gly Ala Gly Phe Gly
50 205 210 215

Ala Val Ile Thr Val Val Ile Val Val Ile Ile Lys Cys Phe Cys
220 225 230 235

55 Lys His Arg Ser Cys Phe Arg Arg Asn Glu Ala Ser Arg Glu Thr Asn
240 245 250

-123-

Asn Ser Leu Thr Phe Gly Pro Glu Glu Ala Leu Ala Glu Gln Thr Val
255 260 265

5 Phe Leu

CLAIMS

1. An isolated nucleic acid comprising a nucleotide sequence encoding a peptide
5 having an activity of a B lymphocyte antigen, B7-2.
2. The isolated nucleic acid of claim 1 which is a cDNA sequence.
3. The isolated nucleic acid of claim 2, wherein the cDNA is of human origin.
10
4. The isolated nucleic acid of claim 3, wherein the cDNA comprises a nucleotide sequence shown in Figure 8 (SEQ ID NO:1).
5. The isolated nucleic acid of claim 3, wherein the cDNA comprises the coding
15 region of a nucleotide sequence shown in Figure 8 (SEQ ID NO:1).
6. The isolated nucleic acid of claim 2, wherein the cDNA is of murine origin.
7. The isolated nucleic acid of claim 6, wherein the cDNA comprises a
20 nucleotide sequence shown in Figure 14 (SEQ ID NO:22).
8. The isolated nucleic acid of claim 6, wherein the cDNA comprises the coding region of a nucleotide sequence shown in Figure 14 (SEQ ID NO:22).
- 25 9. The isolated nucleic acid of claim 1, wherein the peptide comprises an amino acid sequence shown in Figure 8 (SEQ ID NO:2).
10. The isolated nucleic acid of claim 1, wherein the peptide comprises an amino acid sequence shown in Figure 14 (SEQ ID NO:23).
30
11. The isolated nucleic acid of claim 1, wherein the peptide is at least 50% homologous with a sequence comprising an amino acid sequence of Figure 8 (SEQ ID NO:2).
- 35 12. The isolated nucleic acid of claim 1, wherein the peptide is encoded by a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid which encodes a peptide comprising an amino acid sequence shown in Figure 8 (SEQ ID NO:2).

-125-

13. The isolated nucleic acid of claim 1, wherein the peptide is at least 20 amino acid residues in length.

5 14. The isolated nucleic acid of claim 1, wherein the peptide is at least 50% homologous with a sequence comprising an amino acid sequence of Figure 14 (SEQ ID NO:23).

10 15. The isolated nucleic acid of claim 1, wherein the peptide is encoded by a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid which encodes a peptide comprising an amino acid sequence shown in Figure 14 (SEQ ID NO:23).

16. The isolated nucleic acid of claim 15, wherein the peptide is at least 20 amino acid residues in length.

15 17. The isolated nucleic acid of claim 1, wherein the peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

20 18. An isolated DNA comprising a nucleotide sequence encoding a peptide having an activity of a B lymphocyte antigen, B7-2, the peptide having an amino acid sequence represented by formula X_n-Y-Z_m , wherein Y comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2), wherein X_n is amino acid residues selected from amino acid residues contiguous to the amino terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2), wherein Z_m is amino acid residues selected from amino acid residues contiguous to the carboxy terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2), wherein $n=0-23$ and wherein $m=0-84$.

25 19. The isolated DNA of claim 18, wherein $n=0$ and $m=0$.

30 20. The isolated DNA comprising a nucleotide sequence encoding a peptide of at least 20 amino acid residues or more in length and having at least about 50% homology with an amino acid sequence comprising a sequence shown in Figure 8 (SEQ ID NO:2).

35 21. An isolated nucleic acid encoding a B7-2 fusion protein comprising a nucleotide sequence encoding a first peptide having a B7-2 activity and a nucleotide sequence encoding a second peptide corresponding to a moiety that alters the solubility, binding affinity or valency of the first peptide.

-126-

22. The isolated nucleic acid of claim 21 which is a DNA.
23. The isolated nucleic acid of claim 22, wherein the first peptide comprises an extracellular domain of a human B7-2 protein.
5
24. The isolated nucleic acid of claim 23, wherein the first peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).
- 10 25. The isolated nucleic acid of claim 23, wherein the first peptide comprises a variable region-like domain of human B7-2.
- 15 26. The isolated nucleic acid of claim 23, wherein the first peptide comprises a constant region-like domain of human B7-2.
27. The isolated nucleic acid of claim 22, wherein the second peptide comprises an immunoglobulin constant region.
15
28. The isolated nucleic acid of claim 27, wherein the immunoglobulin constant region is a Cy1 domain, including the hinge, CH2 and CH3 region.
20
29. The isolated nucleic acid of claim 27, wherein the immunoglobulin constant region is modified to reduce constant region-mediated biological effector functions.
25
30. The isolated nucleic acid of claim 29, wherein the biological effector function is selected from the group consisting of complement activation, Fc receptor interaction, and complement activation and Fc receptor interaction.
31. The isolated nucleic acid of claim 30, wherein the immunoglobulin constant region is a Cy4 domain, including the hinge, CH2 and CH3 region.
30
32. The isolated nucleic acid of claim 31, wherein at least one amino acid residue of the CH2 domain is modified by substitution, addition or deletion.
35
33. An isolated B7-2 fusion protein comprising a first peptide having a B7-2 activity and a second peptide corresponding to a moiety that alters the solubility, binding affinity or valency of the first peptide.

-127-

34. The isolated B7-2 fusion protein of claim 33, wherein the first peptide comprises an extracellular domain of human B7-2 protein.

5 35. The isolated B7-2 fusion protein of claim 34, wherein the first peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

36. The isolated B7-2 fusion protein of claim 34, wherein the first peptide comprises a variable region-like domain of human B7-2.

10 37. The isolated B7-2 fusion protein of claim 34, wherein the first peptide comprises a constant region-like domain of human B7-2.

15 38. The isolated B7-2 fusion protein of claim 33, wherein the second peptide comprises an immunoglobulin constant region.

39. The isolated B7-2 fusion protein of claim 38, wherein the immunoglobulin constant region is a C_y1 domain, including the hinge, CH2 and CH3 region.

20 40. The isolated B7-2 fusion protein of claim 38, wherein the immunoglobulin constant region is modified to reduce constant region-mediated biological effector functions.

25 41. The isolated B7-2 fusion protein of claim 40, wherein the biological effector function is selected from the group consisting of complement activation, Fc receptor interaction, and complement activation and Fc receptor interaction.

42. The isolated B7-2 fusion protein of claim 41, wherein the immunoglobulin constant region is a C_y4 domain, including the hinge, CH2 and CH3 region.

30 43. The isolated B7-2 fusion protein of claim 42, wherein at least one amino acid residue of the CH2 domain is modified by substitution, addition or deletion.

44. A composition suitable for pharmaceutical administration comprising a fusion protein of claim 33 and a pharmaceutically acceptable carrier.

35 45. A composition suitable for pharmaceutical administration comprising a fusion protein of claim 34 and a pharmaceutically acceptable carrier.

-128-

46. A composition suitable for pharmaceutical administration comprising a fusion protein of claim 36 and a pharmaceutically acceptable carrier.
- 5 47. A composition suitable for pharmaceutical administration comprising a fusion protein of claim 38 and a pharmaceutically acceptable carrier.
48. A recombinant expression vector comprising a nucleic acid of claim 1.
- 10 49. The recombinant expression vector of claim 48, wherein the nucleic acid is a cDNA sequence.
50. The recombinant expression vector of claim 49, wherein the cDNA is of human origin and comprises a nucleotide sequence shown in Figure 8 (SEQ ID NO:1).
- 15 51. The recombinant expression vector of claim 49 which is a plasmid.
52. A recombinant expression vector comprising a nucleic acid of claim 7.
- 20 53. A host cell transfected with the expression vector of claim 48 capable of directing the expression of a peptide having an activity of a B lymphocyte antigen, B7-2.
54. A host cell transfected with the expression vector of claim 50 capable of directing the expression of a peptide having an activity of a B lymphocyte antigen, B7-2.
- 25 55. A host cell transfected with the expression vector of claim 52 capable of directing the expression of a peptide having an activity of a B lymphocyte antigen, B7-2.
56. An isolated, recombinant peptide having an activity of a B lymphocyte antigen, B7-2, expressed by a host cell of claim 54.
- 30 57. A cell transfected with a nucleic acid encoding a peptide having an activity of a B lymphocyte antigen, B7-2, in a form suitable for expression of the peptide on the cell surface.
- 35 58. The cell of claim 57, wherein the nucleic acid is a cDNA comprising a nucleotide sequence shown in Figure 8 (SEQ ID NO:1) in a recombinant expression vector.

59. A tumor cell which is modified to express a T cell costimulatory molecule, B7-2.
- 5 60. The tumor cell of claim 59 which is transfected with a nucleic acid encoding human B7-2 in a form suitable for expression of B7-2.
- 10 61. The tumor cell of claim 59 which is stimulated to express B7-2.
- 15 62. The tumor cell of claim 59 which has a human B7-2 antigen coupled to the tumor cell.
63. The tumor cell of claim 59 which expresses a T cell costimulatory molecule, B7-1.
- 15 64. The tumor cell of claim 59 which expresses a T cell costimulatory molecule, B7-3.
- 20 65. The tumor cell of claim 59 which expresses an MHC class I molecule.
66. The tumor cell of claim 59 which expresses an MHC class II molecule.
- 25 67. The tumor cell of claim 59 which normally expresses an MHC class II associated protein, the invariant chain, and wherein expression of the invariant chain is inhibited.
- 30 68. A tumor cell transfected with a nucleic acid encoding a T cell costimulatory molecule, B7-2, in a form suitable for expression of B7-2.
- 35 69. The tumor cell of claim 68, wherein the nucleic acid is a cDNA in a recombinant expression vector.
70. The tumor cell of claim 68, further transfected with a nucleic acid encoding a T cell costimulatory molecule, B7-1, in a form suitable for expression of B7-1.
71. The tumor cell of claim 68, further transfected with a nucleic acid encoding a T cell costimulatory molecule, B7-3, in a form suitable for expression of B7-3.

-130-

72. The tumor cell of claim 68, further transfected with at least one nucleic acid comprising DNA encoding:

- (a) at least one MHC class II α chain protein; and
- 5 (b) at least one MHC class II β chain protein,

wherein the nucleic acid is in a form suitable for expression of the MHC class II α chain protein(s) and the MHC class II β chain protein(s).

73. The tumor cell of claim 72 which does not express MHC class II molecules
10 prior to transfection of the tumor cell.

74. The tumor cell of claim 68, further transfected with at least one nucleic acid encoding at least one MHC class I α chain protein in a form suitable for expression of the MHC class I protein(s).

15 75. The tumor of claim 74, further transfected with a nucleic acid encoding a β -2 microglobulin protein in a form suitable for expression of the β -2 microglobulin protein.

20 76. The tumor cell of claim 68 which normally expresses an MHC class II associated protein, the invariant chain, and wherein expression of the invariant chain is inhibited.

25 77. The tumor cell of claim 76, wherein expression of the invariant chain is inhibited by transfection of the tumor cell with a nucleic acid which is antisense to a regulatory or a coding region of the invariant chain gene.

78. The tumor cell of claim 68 which is a sarcoma.

79. The tumor cell of claim 68 which is a lymphoma.

30 80. The tumor cell of claim 68 which is selected from a group consisting of a melanoma, a neuroblastoma, a leukemia and a carcinoma.

-131-

81. A method of treating a subject with a tumor, comprising:
 - (a) obtaining tumor cells from the subject;
 - (b) transfecting the tumor cells with a nucleic acid encoding B7-2 in a form suitable for expression of B7-2; and
 - 5 (c) administering the tumor cells to the subject.
82. The method of claim 81, wherein the tumor cells are further transfected with a nucleic acid encoding B7-1.
- 10 83. The method of claim 81, wherein the tumor cells are further transfected with at least one nucleic acid encoding at least one MHC class II α chain protein and at least one MHC class II β chain protein in a form suitable for expression of the MHC class II α chain protein(s) and the MHC class II β chain protein(s).
- 15 84. The method of claim 81, wherein the tumor cells are further transfected with at least one nucleic acid encoding at least one MHC class I α chain protein in a form suitable for expression of the MHC class I protein(s).
- 20 85. The method of claim 84, wherein the tumor cells are further transfected with a nucleic acid encoding a β -2 microglobulin protein in a form suitable for expression of the β -2 microglobulin protein.
- 25 86. The method of claim 81, wherein expression of an MHC class II associated protein, the invariant chain, is inhibited in the tumor cells.
87. The method of claim 86, wherein expression of the invariant chain is inhibited in the tumor cells by transfection of the tumor cell with a nucleic acid which is antisense to a regulatory or a coding region of the invariant chain gene.
- 30 88. The method of claim 81, wherein the tumor is a sarcoma.
89. The method of claim 81, wherein the tumor is a lymphoma.
- 35 90. The method of claim 81, wherein the tumor is selected from a group consisting of a melanoma, a neuroblastoma, a leukemia and a carcinoma.

-132-

91. A method of inducing an anti-tumor response by CD4+ T lymphocytes in a subject with a tumor, comprising:

- (a) obtaining tumor cells from the subject;
- (b) transfecting the tumor cells with at least one nucleic acid comprising DNA encoding:

- (i) B7-2,
- (ii) an MHC class II α chain protein, and
- (iii) an MHC class II β chain protein,

wherein the nucleic acid is in a form suitable for expression of B7-2, the MHC class II α chain protein and the MHC class II β chain protein; and

- (c) administering the tumor cells to the subject.

92. A method for treating a subject with a tumor comprising modifying tumor cells *in vivo* to express a T cell costimulatory molecule, B7-2.

- 15 93. The method of claim 92, wherein tumor cells are modified *in vivo* by delivering to the subject *in vivo* a nucleic acid encoding B7-2 in a form suitable for expression of B7-2.
- 20 94. The method of claim 93, wherein the nucleic acid is delivered to the subject *in vivo* by injection of the nucleic acid in an appropriate vehicle into the tumor.

- 25 95. A method for treating a subject with a tumor, comprising:
 - (a) obtaining tumor cells and T lymphocytes from the subject;
 - (b) culturing the T lymphocytes from the subject *in vitro* with the tumor cells from the subject and with a stimulatory form of B7-2; and
 - (c) administering the T lymphocytes to the subject.

- 30 96. A peptide having an activity of a B lymphocyte antigen, B7-2, produced by recombinant expression of a nucleic acid of claim 1.

- 97. A peptide having an activity of a B lymphocyte antigen, B7-2, produced by recombinant expression of a nucleic acid of claim 4.

- 35 98. A peptide having an activity of a B lymphocyte antigen, B7-2, produced by recombinant expression of a nucleic acid of claim 5.

-133-

99. A peptide of claim 98 comprising an amino acid sequence set forth in Figure 8 (SEQ ID NO: 2).

100. A peptide having an activity of a B lymphocyte antigen, B7-2, produced by
5 recombinant expression of a DNA of claim 18.

101. A peptide having an activity of a B lymphocyte antigen, B7-2, produced by
recombinant expression of a DNA of claim 20.

10 102. A substantially pure preparation of a peptide having an activity of a B
lymphocyte antigen, B7-2.

15 103. A substantially pure preparation of a peptide having an activity of a B
lymphocyte antigen, B7-3.

104. A peptide having an amino acid sequence represented by a formula $X_n\text{-}Y\text{-}Z_m$,
wherein Y is amino acid residues selected from the group consisting of: amino acid residues
55-68 of the sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 81-89 of the
sequence shown in Figure 8 (SEQ ID NO:2); amino acid residues 128-142 of the sequence
20 shown in Figure 8 (SEQ ID NO:2); amino acid residues 160-169 of the sequence shown in
Figure 8 (SEQ ID NO:2); amino acid residues 188-200 of the sequence shown in Figure 8
(SEQ ID NO:2); and amino acid residues 269-282 of the sequence shown in Figure 8 (SEQ
ID NO:2), wherein X_n is amino acid residues selected from amino acid residues contiguous
to the amino terminus of Y in the sequence shown in Figure 8 (SEQ ID NO:2), wherein Z_m is
25 amino acid residues selected from amino acid residues contiguous to the carboxy terminus of
Y in the sequence shown in Figure 8 (SEQ ID NO:2), wherein n=0-30 and wherein m=0-30.

105. A peptide of claim 104, wherein n=0 and m=0.

30 106. An antibody specifically reactive with a peptide produced by recombinant
expression of a nucleotide sequence encoding a peptide having an activity of a human B
lymphocyte antigen, B7-2.

35 107. The antibody of claim 106, wherein the nucleotide sequence comprises a
coding region of a nucleotide sequence shown in Figure 8 (SEQ ID NO:1).

108. The antibody of claim 106 which is a monoclonal antibody.

-134-

109. The antibody of claim 108 which is an IgG1 antibody.
110. The antibody of claim 108 which is an IgG2a antibody.
5
111. A hybridoma HF2.3D1 designated by ATCC Accession No. _____.
10
112. A monoclonal antibody produced by the hybridoma of claim 111.
113. A hybridoma HA5.2B7 designated by ATCC Accession No. _____.
15
114. A monoclonal antibody produced by the hybridoma of claim 113.
115. A hybridoma HA3.1F9 designated by ATCC Accession No. _____.
116. A monoclonal antibody produced by the hybridoma of claim 115.
20
117. A nonhuman, transgenic animal which contains cells transfected to express a peptide having an activity of a B lymphocyte antigen, B7-2.
118. The nonhuman, transgenic animal of claim 117 which is a mouse.
25
119. A nonhuman, knockout animal which contains cells having an altered gene encoding a B lymphocyte antigen, B7-2.
120. The nonhuman, knockout animal of claim 119 which is a mouse.
30
121. A composition suitable for pharmaceutical administration comprising a peptide having an activity of a B lymphocyte antigen, B7-2, and a pharmaceutically acceptable carrier.
122. The composition of claim 121 further comprising a peptide having an activity of a B lymphocyte antigen, B7-1.
35
123. The composition of claim 121, wherein the peptide comprises an amino acid sequence set forth in Figure 8 (SEQ ID NO: 2).

-135-

124. The composition of claim 123, wherein the peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

125. A method for producing a peptide having an activity of a B lymphocyte antigen, B7-2, comprising culturing a host cell of claim 53 in a medium to express the peptide and isolating the peptide from the medium.

126. A method for producing a peptide having an activity of a B lymphocyte antigen, B7-2, comprising culturing a host cell of claim 54 in a medium to express the peptide and isolating the peptide from the medium.

127. A method for inhibiting an interaction of a B lymphocyte antigen, B7-2, with its natural ligand(s) on the surface of immune cells, comprising contacting an immune cell with a reagent which inhibits B7-2 binding with its natural ligand(s), to thereby inhibit costimulation of the immune cell through the B7-2-ligand interaction.

128. The method of claim 126, wherein the reagent is a peptide having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to the immune cell.

129. The method of claim 128, wherein the peptide is a soluble, monomeric peptide.

130. The method of claim 129, wherein the peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

131. The method of claim 130, wherein the reagent is a B7-2 fusion protein comprising a first peptide having B7-2 activity and a second peptide comprising a moiety that alters the solubility, binding affinity or valency of the first peptide.

132. The method of claim 131, wherein the first peptide comprises an extracellular domain of the human B7-2 protein.

133. The method of claim 132, wherein the first peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

134. The method of claim 131, wherein the second peptide comprises an immunoglobulin constant region.

-136-

135. The method of claim 134, wherein the immunoglobulin constant region is a Cy 1 domain, including the hinge, CH2 and CH3 region.

5 136. The method of claim 131, wherein the reagent is an antibody reactive with B7-2.

137. The method of claim 136, wherein the antibody is a monoclonal antibody.

10 138. A method for downregulating T cell mediated immune responses in a subject, comprising administering to the subject an agent having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to T cells, in an amount effective to inhibit T cell proliferation and/or cytokine secretion in the subject.

15 139. The method of claim 138, wherein the agent is a peptide having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to T cells.

140. The method of claim 138, wherein the agent is an antibody reactive with B7-2.

20 141. The method of claim 140, wherein the antibody is a monoclonal antibody.

142. The method of claim 138, further comprising administering to the subject an agent having B7-1 binding activity, but lacking the ability to deliver a costimulatory signal to T cells.

25 143. The method of claim 142, wherein the agent is a peptide having B7-1 binding activity, but lacking the ability to deliver a costimulatory signal to T cells.

144. The method of claim 142, wherein the agent is an antibody reactive with B7-1.

30 145. The method of claim 144, wherein the antibody is a monoclonal antibody.

146. The method of claim 138, further comprising administering to the subject an immunomodulating reagent selected from the group consisting of an antibody reactive with CD28, an antibody reactive with CTLA4, an antibody reactive with a cytokine, a CTLA4Ig fusion protein, a CD28Ig fusion protein, and an immunosuppressive drug.

-137-

147. A method for treating an autoimmune disease in a subject mediated by interaction of a B lymphocyte antigen, B7-2, with its natural ligand(s) on the surface of immune cells, comprising administering to the subject an inhibitory form of B7-2 protein, to thereby inhibit costimulation of the immune cells through the B7-2-ligand interaction.

5

148. The method of claim 147, wherein the autoimmune disease is selected from the group consisting of diabetes mellitus, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, systemic lupus enthmatoisis, and autoimmune thyroiditis.

10

149. The method of claim 147, wherein the inhibitory form of B7-2 protein is a peptide having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to immune cells.

15

150. The method of claim 149, wherein the peptide is a soluble, monomeric

peptide.

151. The method of claim 150, wherein the peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

20

152. The method of claim 147, wherein the inhibitory form of B7-2 protein is a B7-2 immunoglobulin fusion protein (B7-2Ig) comprising a first peptide comprising an extracellular domain of the B7-2 protein and a second peptide comprising an immunoglobulin constant domain.

25

153. The method of claim 152, wherein the extracellular domain of the B7-2 protein comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

30

154. The method of claim 147, wherein the inhibitory form of B7-2 protein is an antibody reactive with B7-2.

155. The method of claim 154, wherein the antibody is a monoclonal antibody.

35

156. The method of claim 149, further comprising administering to the subject a peptide having B7-1 binding activity, but lacking the ability to deliver a costimulatory signal to T cells.

-138-

157. The method of claim 147, further comprising administering to the subject an immunomodulating reagent selected from the group consisting of an antibody reactive with B7-1, an antibody reactive with CD28, an antibody reactive with CTLA4, an antibody reactive with a cytokine, a CTLA4Ig fusion protein, a CD28Ig fusion protein, and an
5 immuno suppressive drug.

158. A method for treating allergy in a subject mediated by interaction of a B lymphocyte antigen, B7-2, with its natural ligand(s) on the surface of immune cells, comprising administering to the subject an inhibitory form of B7-2 protein, to thereby inhibit
10 costimulation of the immune cells through the B7-2 -ligand interaction.

159. The method of claim 158, wherein the inhibitory form of B7-2 protein is a peptide having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to immune cells.

160. A method for inhibiting donor T cell proliferation and/or cytokine secretion in a transplant recipient to thereby prevent graft-versus-host disease (GVHD) in the recipient, comprising contacting donor T cells to be transplanted with an agent having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to the T cells.
15

161. The method of claim 160, wherein the agent is a peptide having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to T cells.
20

162. The method of claim 161, wherein the peptide comprises amino acid residues
25 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

163. The method of claim 160, wherein the agent is an antibody reactive with B7-2.

164. The method of claim 163, wherein the antibody is a monoclonal antibody.
30

165. A method for inhibiting transplantation rejection in a recipient of a tissue or organ transplant, comprising administering to the recipient an agent having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to the T cells.

35 166. The method of claim 165, wherein the agent is a peptide having B7-2 binding activity, but lacking the ability to deliver a costimulatory signal to T cells.

-139-

167. The method of claim 166, wherein the peptide comprises amino acid residues 24-245 of the sequence shown in Figure 8 (SEQ ID NO:2).

168. The method of claim 165, wherein the agent is an antibody reactive with B7-2.

5

169. The method of claim 168, wherein the antibody is a monoclonal antibody.

170. A method for upregulating T cell mediated immune responses in a subject, comprising administering to the subject a peptide having B7-2 activity, in an amount effective to stimulate T cell proliferation and/or cytokine secretion in the subject.

171. The method of claim 170, further comprising administering to the subject a peptide having B7-1 activity.

172. The method of claim 170, further comprising administering to the subject a pathogen or portion thereof to thereby induce an anti-pathogen immune response in the subject.

173. The method of claim 172, wherein the pathogen is a virus.

20

174. A method of identifying molecules which modulate expression of a B7-2 antigen, comprising

- a) contacting a cell which expresses a peptide having B7-2 activity with a molecule to be tested, under conditions appropriate for interaction of the molecule with the cell; and
- b) determining the effect of the molecule on cell expression of the peptide having B7-2 activity.

175. The method of claim 174, wherein the effect of the molecule on cell expression of the peptide having B7-2 activity is determined by detecting the presence of the peptide on the cell surface.

176. The method of claim 175, wherein the presence of the peptide on the cell surface is detected by immunofluorescence with an antibody reactive with the peptide or with a CTLA4Ig or CD28Ig fusion protein.

35

-140-

177. The method of claim 174, wherein the effect of the molecule on cell expression of the peptide having B7-2 activity is determined by detecting the presence of mRNA encoding the peptide in the cell.

5 178. The method of claim 177, wherein the presence of mRNA is detected by hybridization with B7-2 cDNA.

179. A method of identifying a cytokine produced by an immune cell in response to costimulation with a B7-2 antigen, comprising

- 10 a) contacting an activated immune cell and a cell which expresses a peptide having B7-2 activity, in an appropriate cell culture medium; and
b) determining the presence of a cytokine in the cell culture medium.

180. The method of claim 179, wherein the immune cell is a T cell.

15 181. The method of claim 179, wherein the presence of a cytokine in the cell culture medium is determined by contacting the medium with an antibody reactive with the cytokine.

20 182. A method of identifying molecules which inhibit costimulation of immune cells by a B7-2 antigen, comprising

- a) contacting an immune cell which has received a primary activation signal with a stimulatory form of B7-2 protein and a molecule to be tested, under conditions appropriate for interaction of the molecule with the immune cell and the stimulatory form of B7-2 protein; and
b) determining the effect of the molecule on costimulation of the immune cell by the stimulatory form of B7-2 protein.

183. The method of claim 182, wherein the immune cell is a T cell.

30 184. The method of claim 183, wherein the effect of the molecule on costimulation of the T cell is determined by detecting T cell proliferation and/or cytokine production.

185. The method of claim 182, wherein the stimulatory form of B7-2 is a cell 35 which expresses a peptide having B7-2 activity on the cell surface.

-141-

186. A method of identifying molecules which inhibit binding of a B7-2 antigen to a ligand on the surface of immune cells, comprising

- a) contacting a labeled B7-2 ligand and a molecule to be tested with a peptide having B7-2 activity;
- 5 b) removing unbound labeled B7-2 ligand; and
- c) determining the amount of labeled B7-2 ligand bound to the peptide having B7-2 activity, as an indication of the ability of the molecule to inhibit binding of the B7-2 ligand to a B7-2 antigen.

10 187. The method of claim 186, wherein the immune cell is a T cell and the B7-2 ligand is CTLA4 or CD28.

188. The method of claim 186, wherein the peptide is immobilized on a solid phase support.

15 189. A method of identifying molecules which inhibit intracellular signaling by an immune cell in response to a stimulatory form of a B7-2 protein, comprising

- a) contacting an immune cell which has received a primary activation signal and which expresses a B7-2 ligand on the cell surface with a stimulatory form of B7-2 protein and a molecule to be tested, under conditions appropriate for interaction of the molecule with the immune cell and the stimulatory form of B7-2 protein; and
- 20 b) determining the effect of the molecule on intracellular signaling by the immune cell in response to the stimulatory form of B7-2 protein.

25 190. The method of claim 189, wherein the immune cell is a T cell.

191. The method of claim 190, wherein the effect of the molecule on intracellular signaling by the immune cell is determined by detecting T cell proliferation and/or cytokine production.

30 192. The method of claim 189, wherein the stimulatory form of B7-2 is a cell which expresses a peptide having B7-2 activity on the cell surface.

35 193. A method of isolating a B lymphocyte antigen, B7-3, comprising contacting a cell material which contains a peptide having B7-3 activity, with an antibody reactive with B7-3 under conditions appropriate for binding of the antibody to the peptide and isolating the peptide from the antibody.

-142-

194. The method of claim 193, wherein the antibody is a monoclonal antibody BB-

1.

1 / 27

FIG. 1A

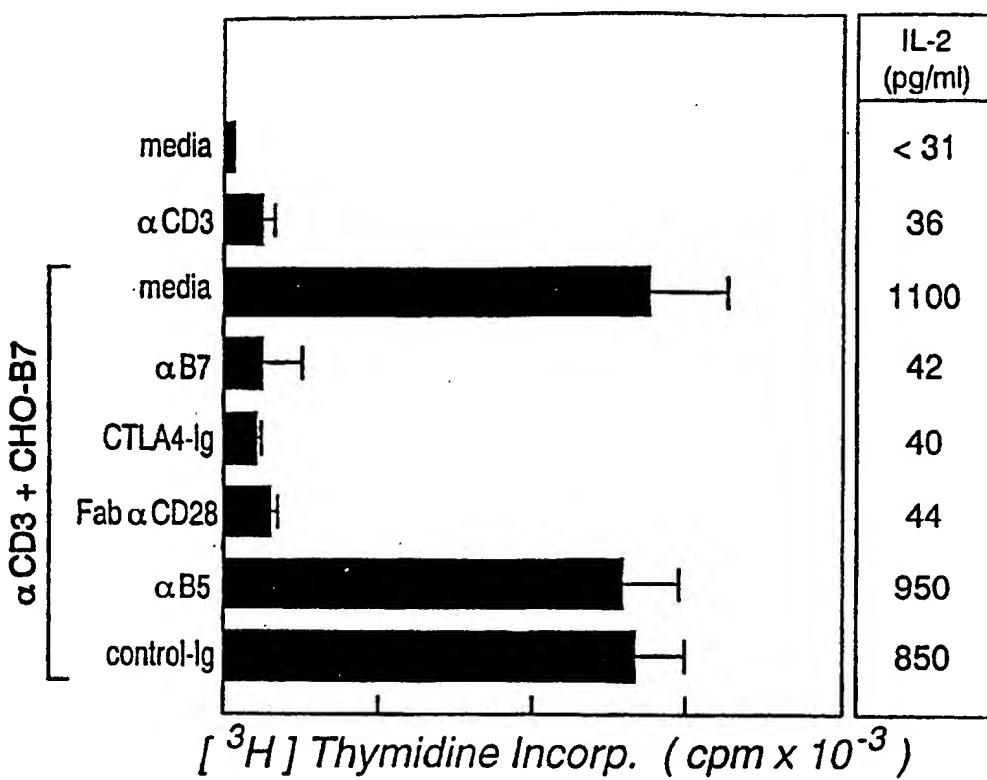
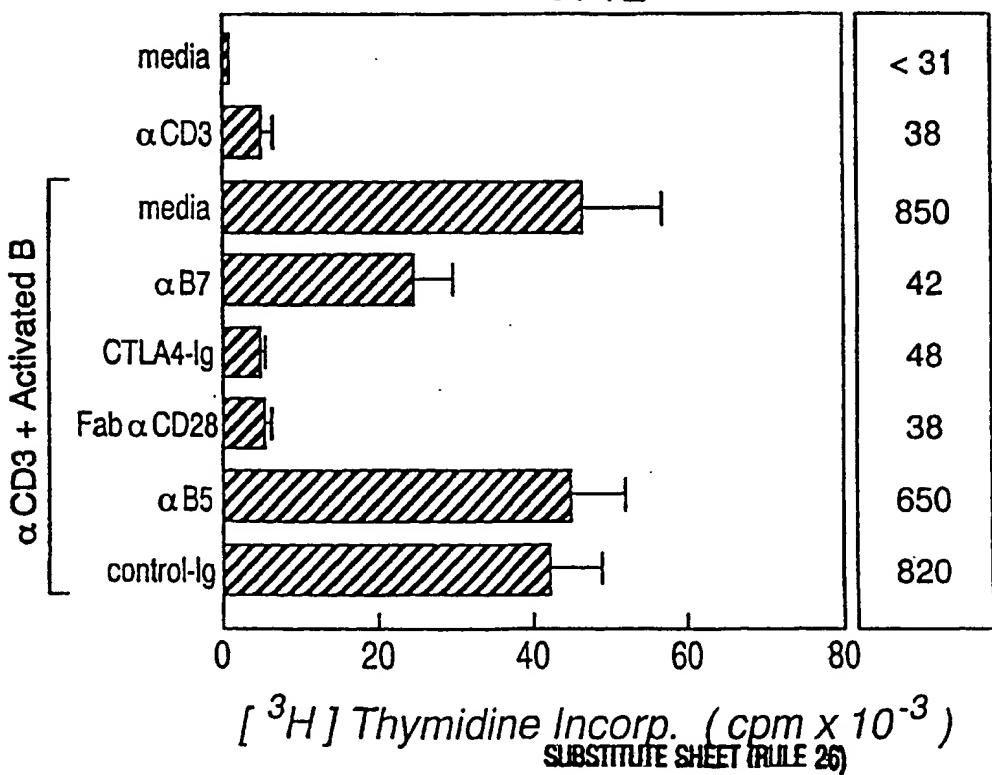


FIG. 1B



2 / 27

FIG. 2C

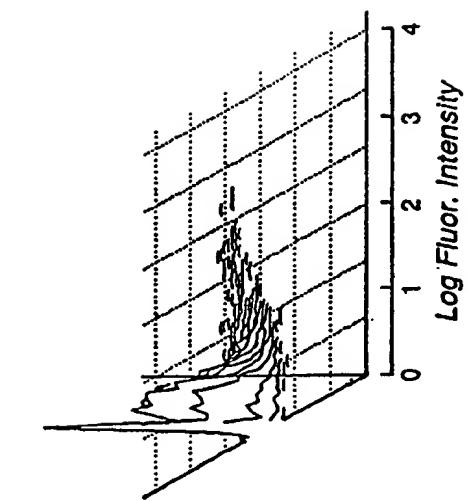


FIG. 2B

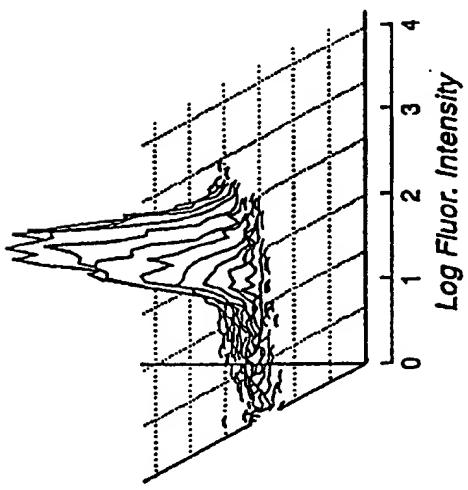
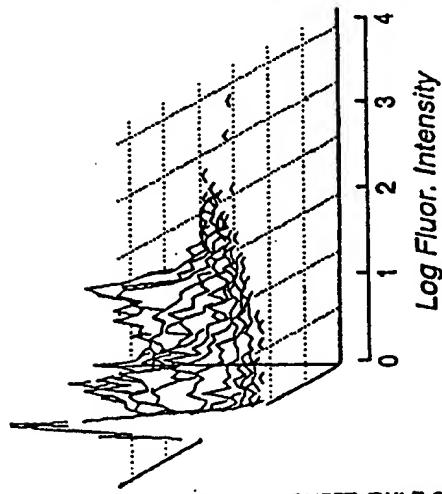


FIG. 2A



SUBSTITUTE SHEET (RULE 26)

FIG. 3A

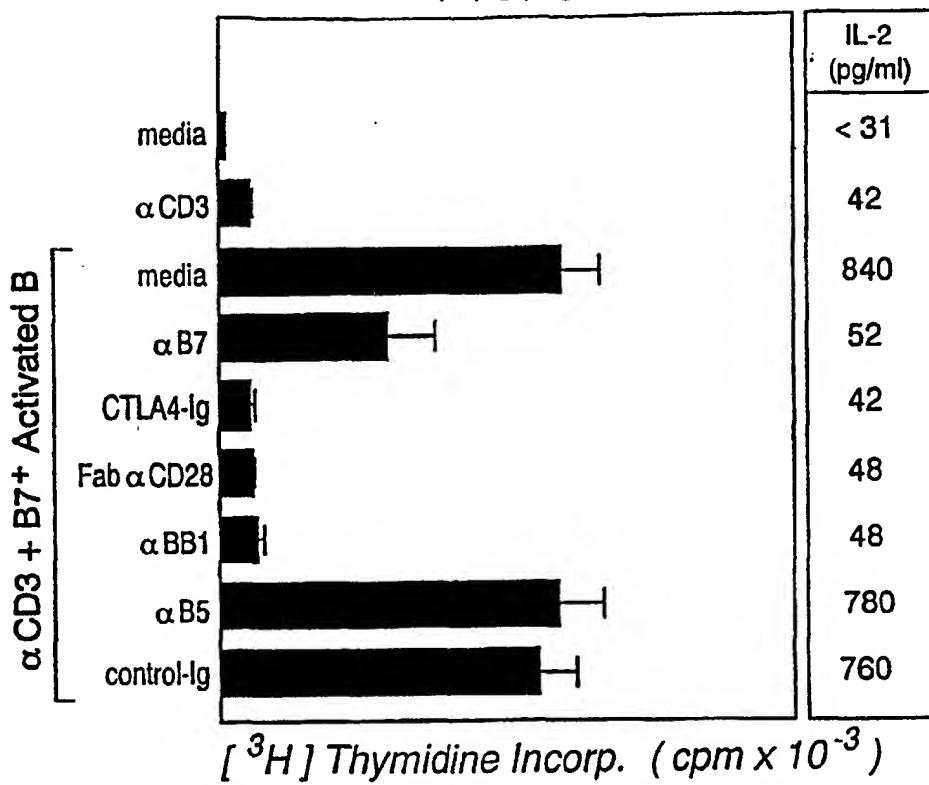
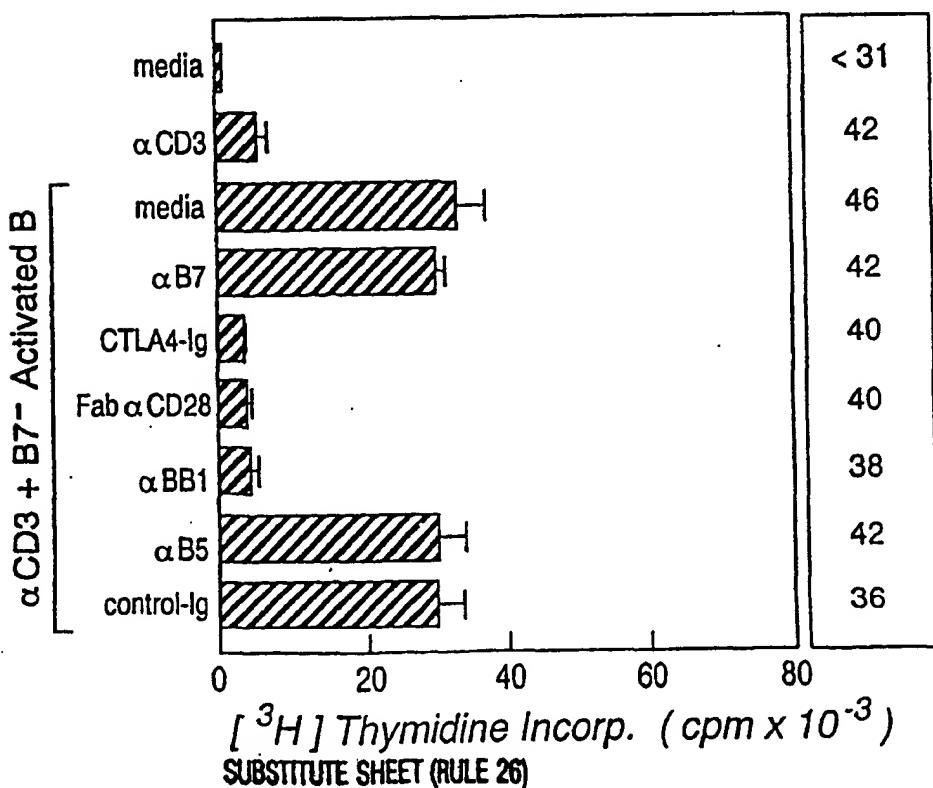


FIG. 3B



4 / 27

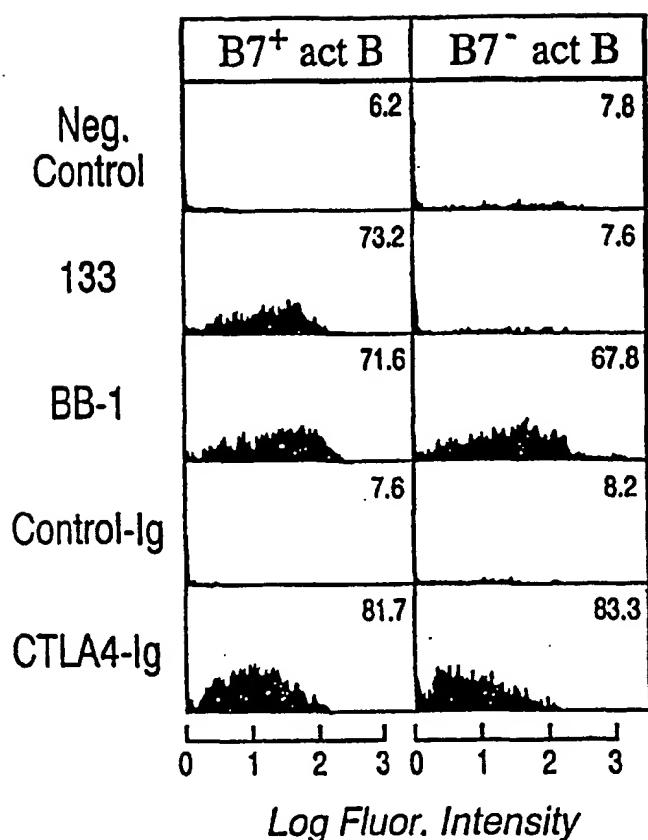


FIG. 4

5 / 27

B Cells Activated by sIg Crosslinking

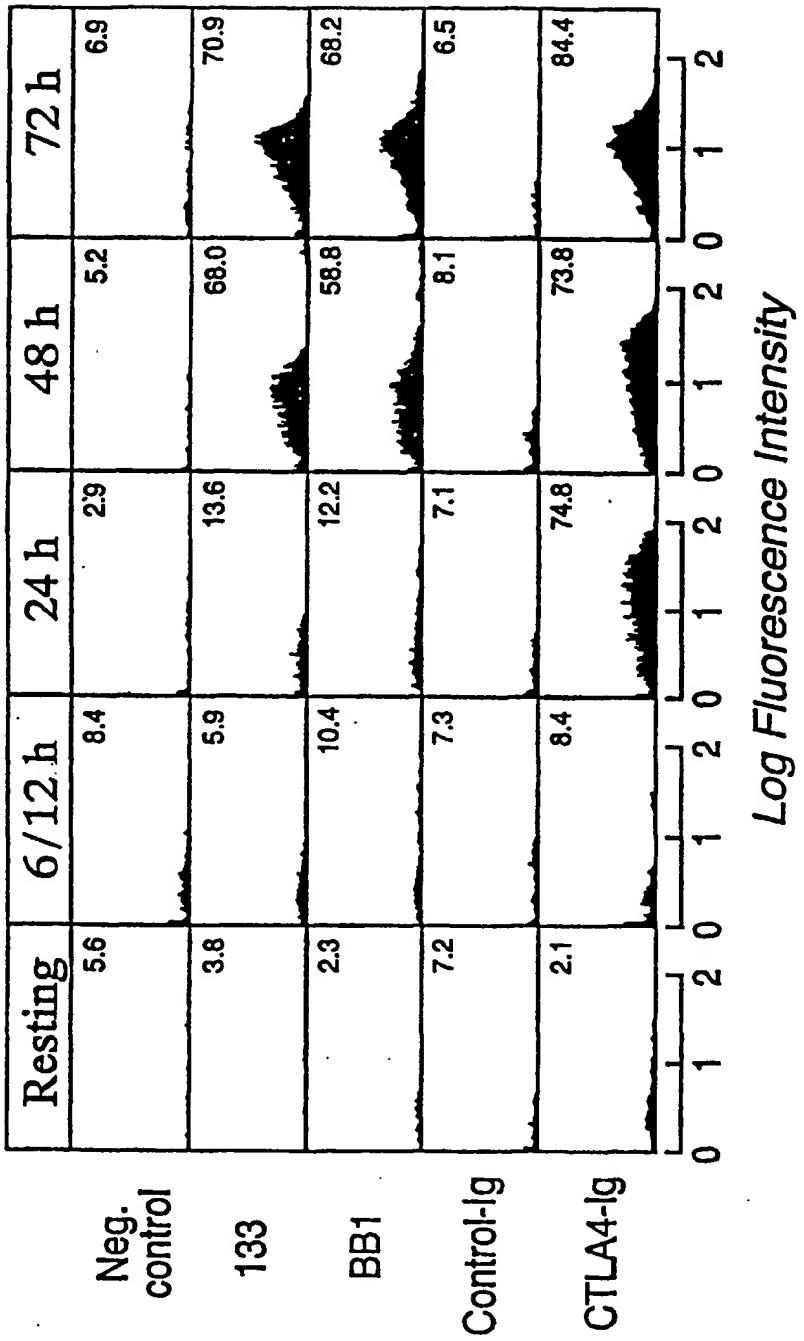
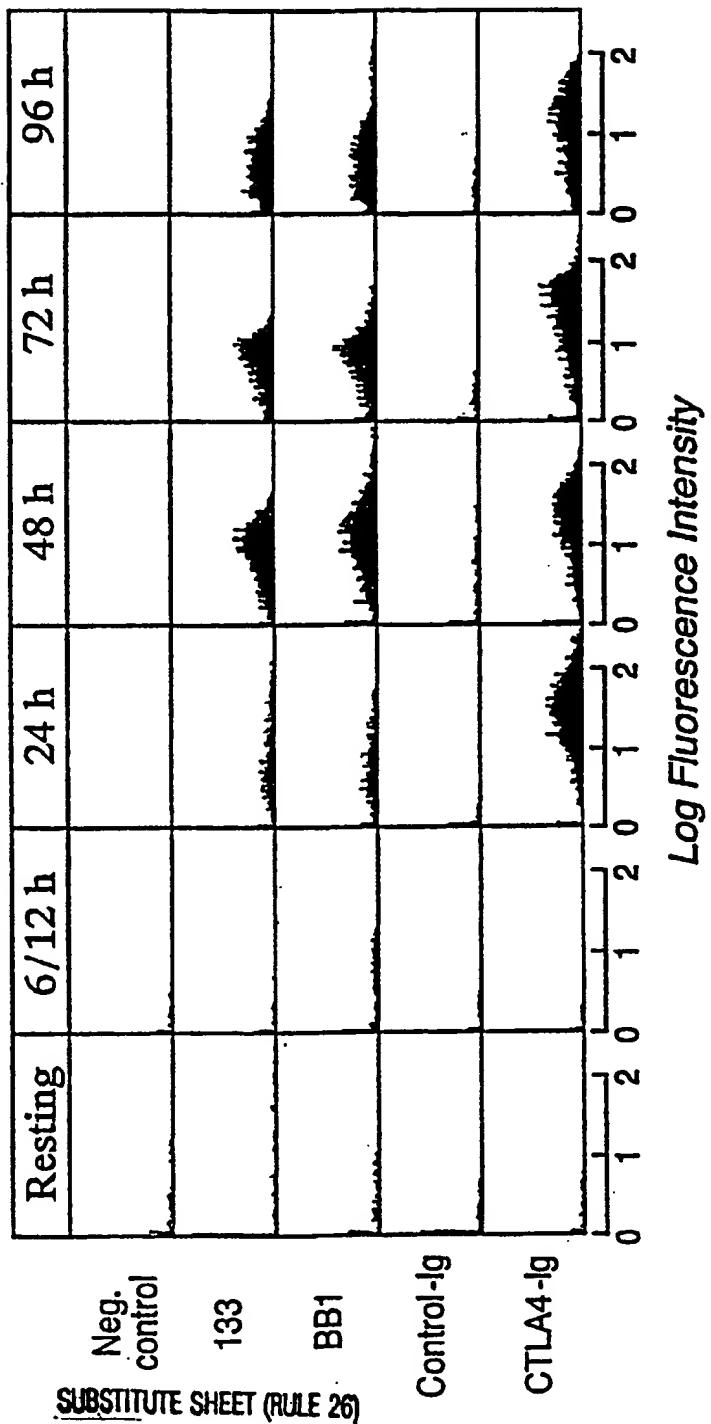


FIG. 5

6 / 27

FIG. 6

B Cells Activated by Class II Crosslinking



SUBSTITUTE SHEET (RULE 26)

FIG. 7A

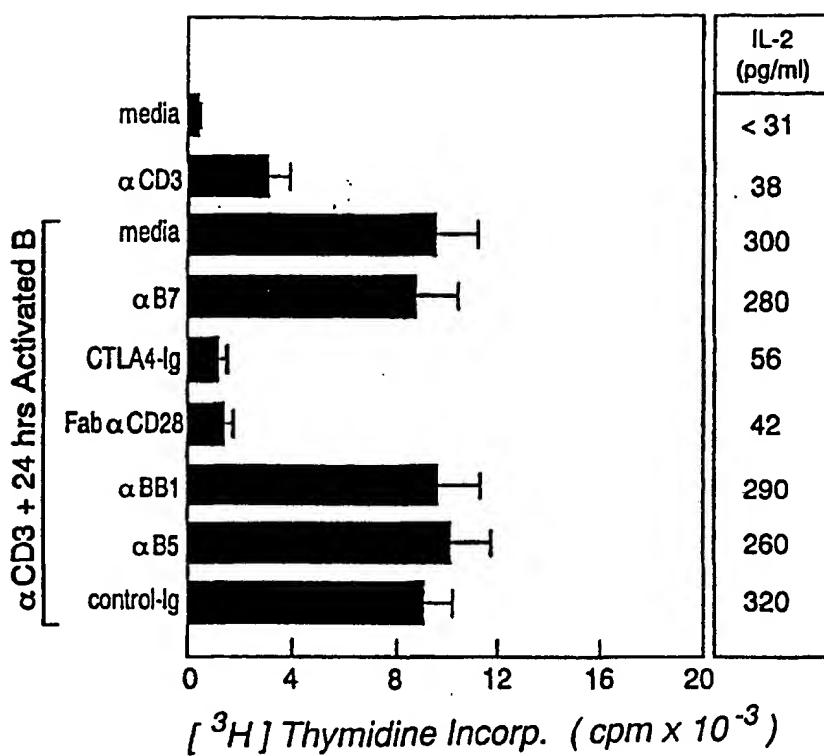
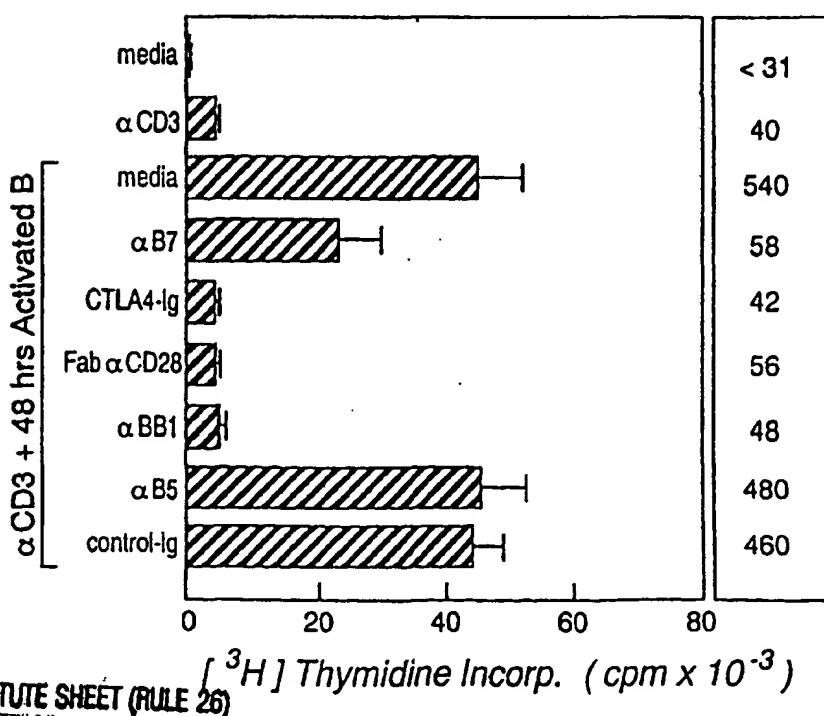


FIG. 7B



SUBSTITUTE SHEET (RULE 26)

8 / 27

FIG. 8A

1 CACAGGGGTGAAAGCTTTCCTCTGCCTGCTAAACAGGGACTAGCCACAGACACACCGATGAGTGCTGGGGTC 70
 71 ATTTCCAGATATTAGCTCACAGCAGAACGCCAAATGGATCCCCAGTGCACATGGACTATGGACTGAACA 140
 M D P Q C T M G L S N 11

141 TTCTCTTGTGATGGCCCTTCCTCTCTGGCTCTGGCTCTGGCTCTGAAAGATTCAGCTTATTTCATGAGAC 210
 12 I L F V M A F L L S G A A P L K I Q A Y F N E T 35
 #
 211 TGCAGACCTGCCATGCCAAACTCTCAAAACCAAGCCTGAGTGAGCTAGTAGTATTGGCAG 280
 36 A D L P C Q F A N S Q N Q S L S E L V F W Q 58
 * #
 281 GACCAGGAAACACTTGGTTCTGAATGAGGTATACTTGGCAAAGAGAAATTGACAGTGGTCATTCCAAGT 350
 59 D Q E N L V L N E V Y L G K E K F D S V H S K 81

351 ATATGGCCGCCACAAGTTTCATTCGGACAGTGGACCCCTGAGACTTCACAATCTTCAGATCAAGGACAA 420
 82 Y M G R T S F D S D S W T L R L H N L Q I K D K 105

SUBSTITUTE SHEET (RULE 26)

9 / 27

FIG. 8B

421 GGGCTGATCAATGTTATCATCCATCACAAAAGCCACAGGAATGATTGGCATCCACCAGATGAATTCT 490

106 G L Y Q C I I H H K K P T G M I R I H Q M N S 128
* #

491 GAACTGTCAAGTGTCTTAACCTCAGTCACCTGAATAATAGTACCAATTCTTAACAGAAAATGTTGT 560

129 E L S V L A N F S Q P E I V P I S N I T E N V 151
#

561 ACATAAATTGACCTGCTCATCTATACACGGTTACCCAGAACCTAAGAAGATGAGTGTTTGCTAAGAAC 630

152 Y I N L T C S S I H G Y P E P K M S V L L R T 175
*

631 CAAGAAATTCAACTATCCAGTATGTTATATTATCGAGATAATCTCAAGATAATGTCACAGAACTGTACGAC 700

176 K N S T I E Y D G I M Q K S Q D N V T E L Y D 198
#

701 GTTCCCATCAGCTTGTCTGTTCAATTCCCTGATGTTACGGAAATATGACCATCTCTGTATTCGGAAA 770

199 V S I S L S V S F P D V T S N M T I F C I L E 221
*

771 CTGACAAGACCCGCTTTATCTCACCTTCTATAGAGCTTGAGGACCCCTCAGGCCCTCCCCAGACCA 840

222 T D K T R L L S S P F S I E L E D P Q P P D H 245

SUBSTITUTE SHEET (RULE 26)

10 / 27

FIG. 8C

841 CATTCCCTGGATTACAGCTGTA
CTTCCAAACAGTTATTATGTGATGGTTCTGTCTAATTCTATGG 910

246 I P W I T A V L P T V I I C V M V F C L I L W 268

911 AAATGGAAGAACAGGGCTCGCAACTCTTATAAATGTGGAAACCAACACAATGGAGGGAAAGAGA 980

269 K W K K R P R N S Y K C G T N T M E R E E 291

981 GTGAAACAGACCAAGAAAAGAGAAAATCCATATACTGAAAGATCTGATGAAGCCCCAGCGTGTTTAA 1050

292 S E Q T K R E K I H I P E R S D E A Q R V F K 315

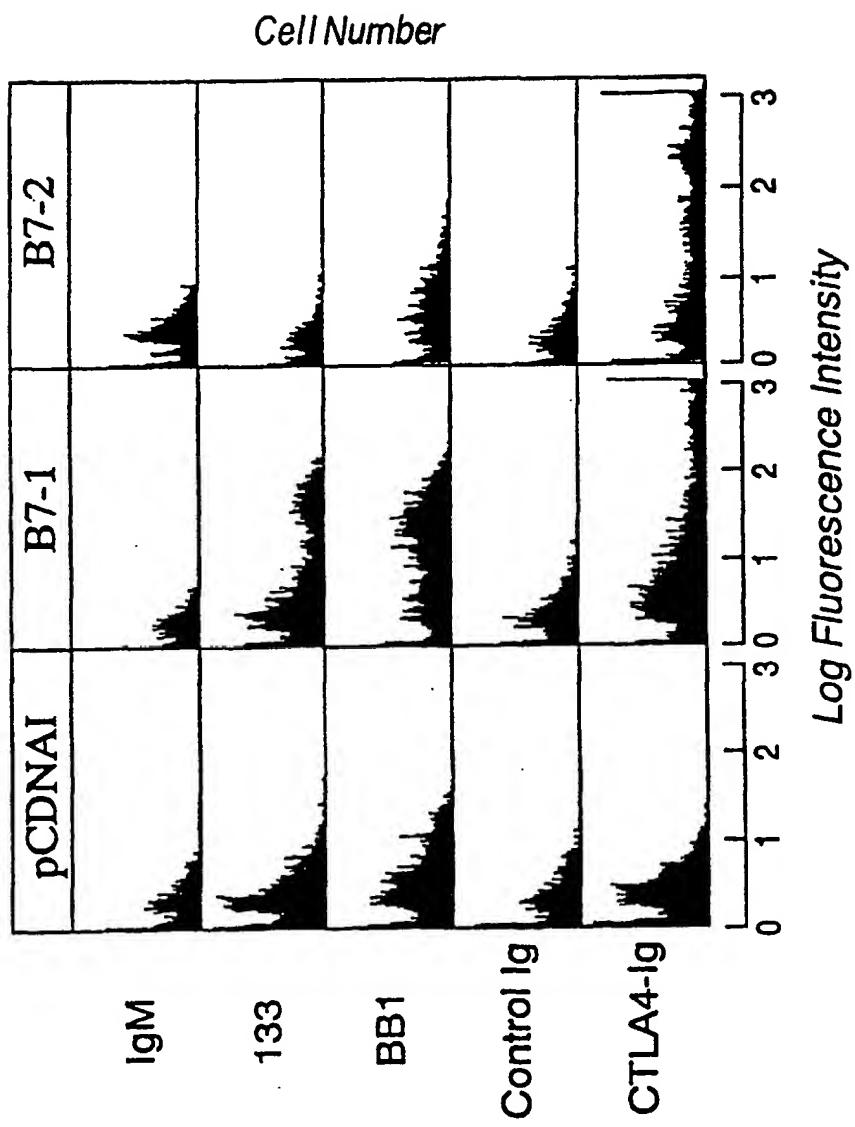
1051 AAGTTTCGAAAGACATCTTICATGCCGACAAAAAGTGATACATGTTTTAATTAAAGAGTAAGCCC
AAAAAA 1120

316 S S K T S S C D K S D T C F * 329

SUBSTITUTE SHEET (RULE 26)

11 / 27

FIG. 9



12 / 27

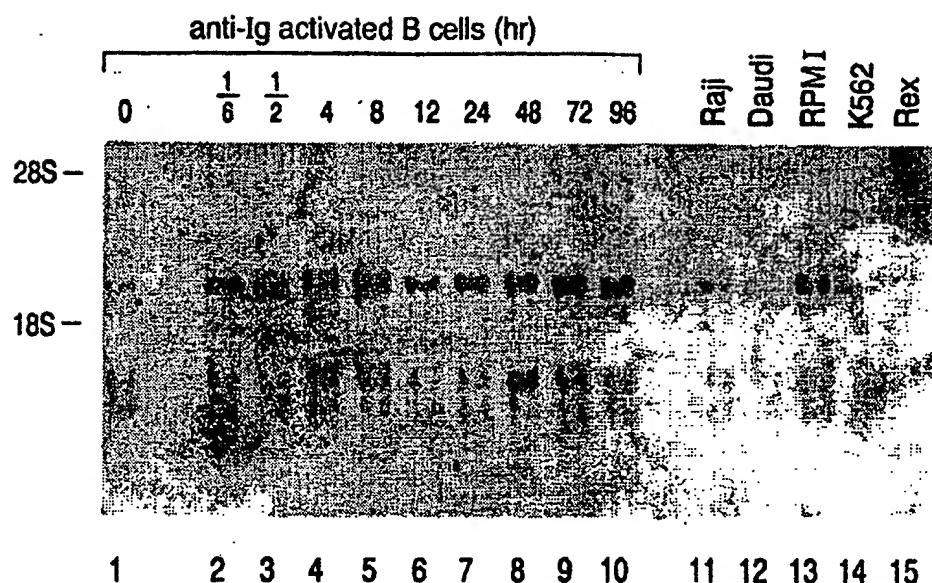


FIG. 10A

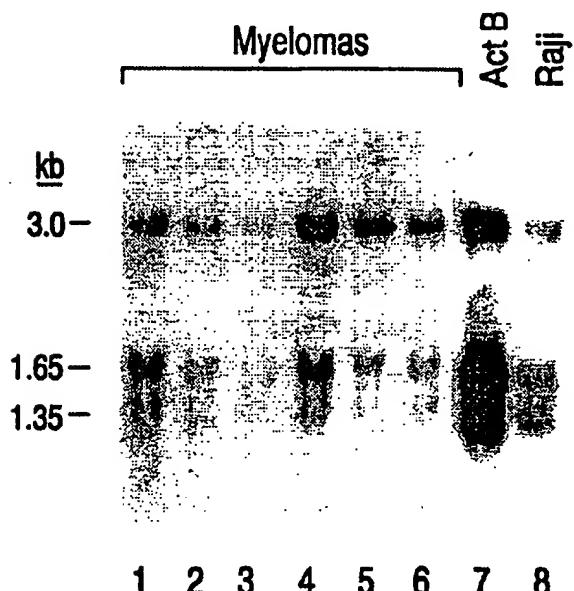


FIG. 10B

SUBSTITUTE SHEET (RULE 26)

13 / 27

FIG. IIA

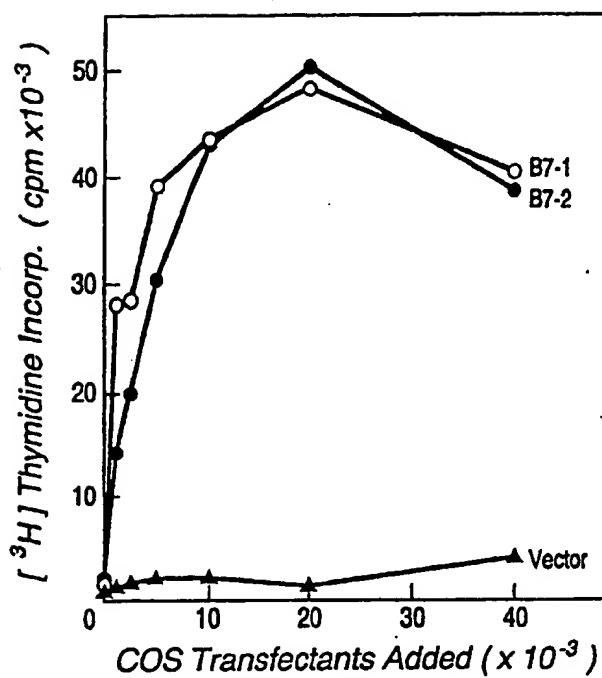
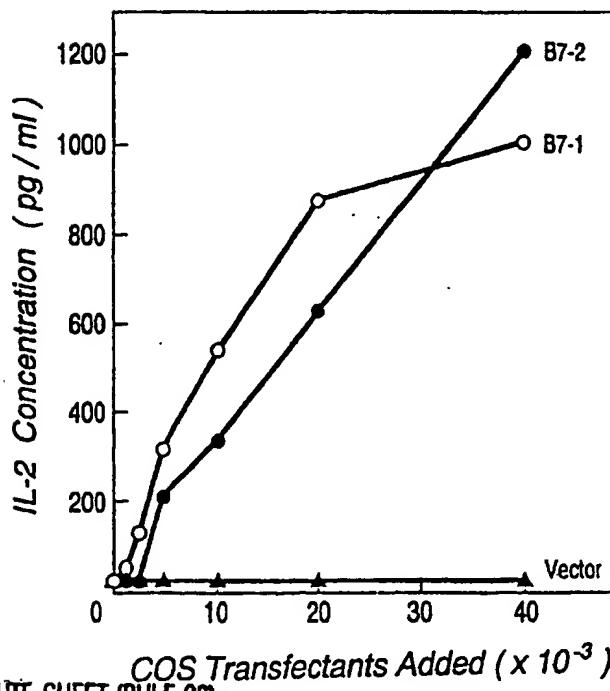
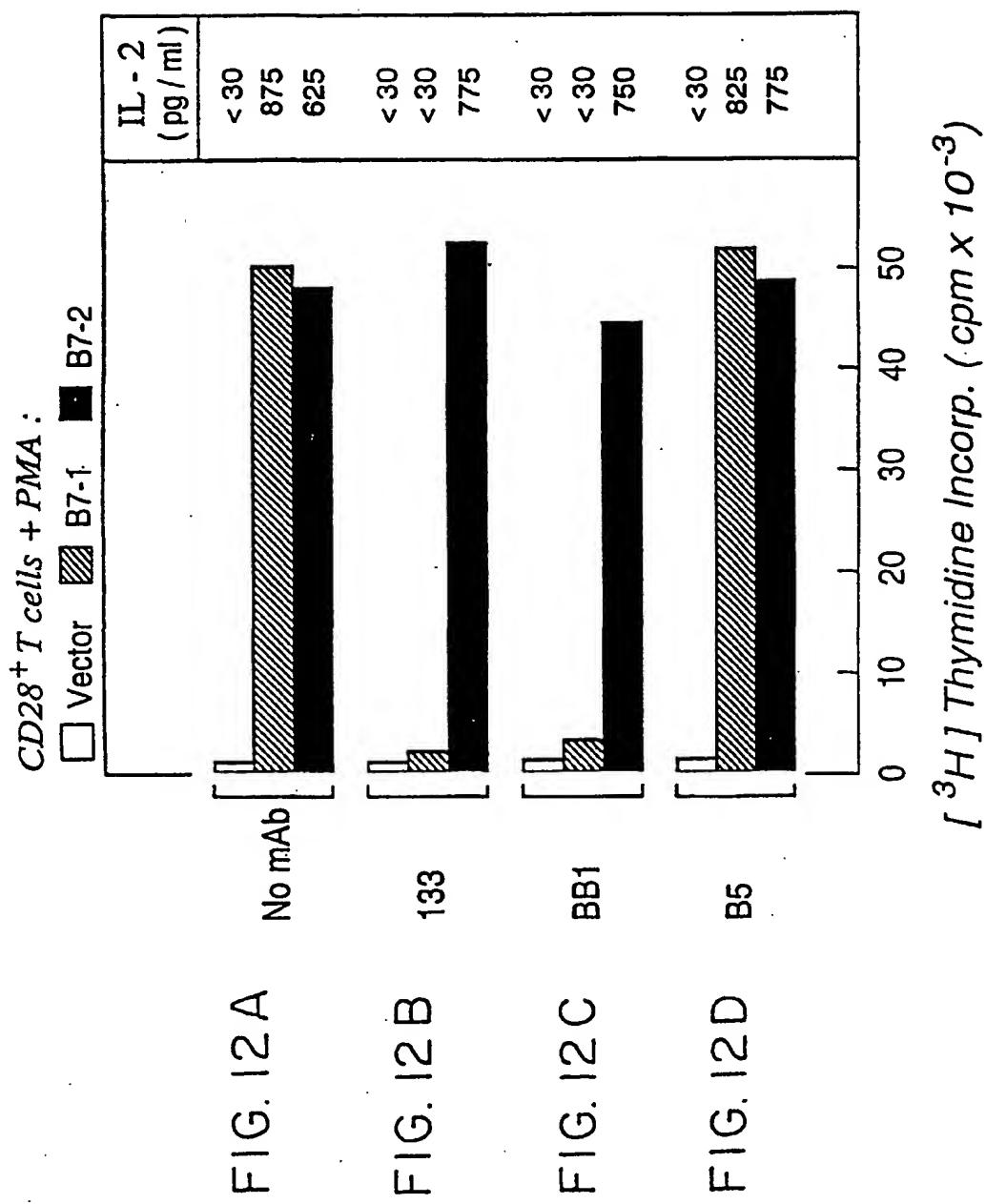


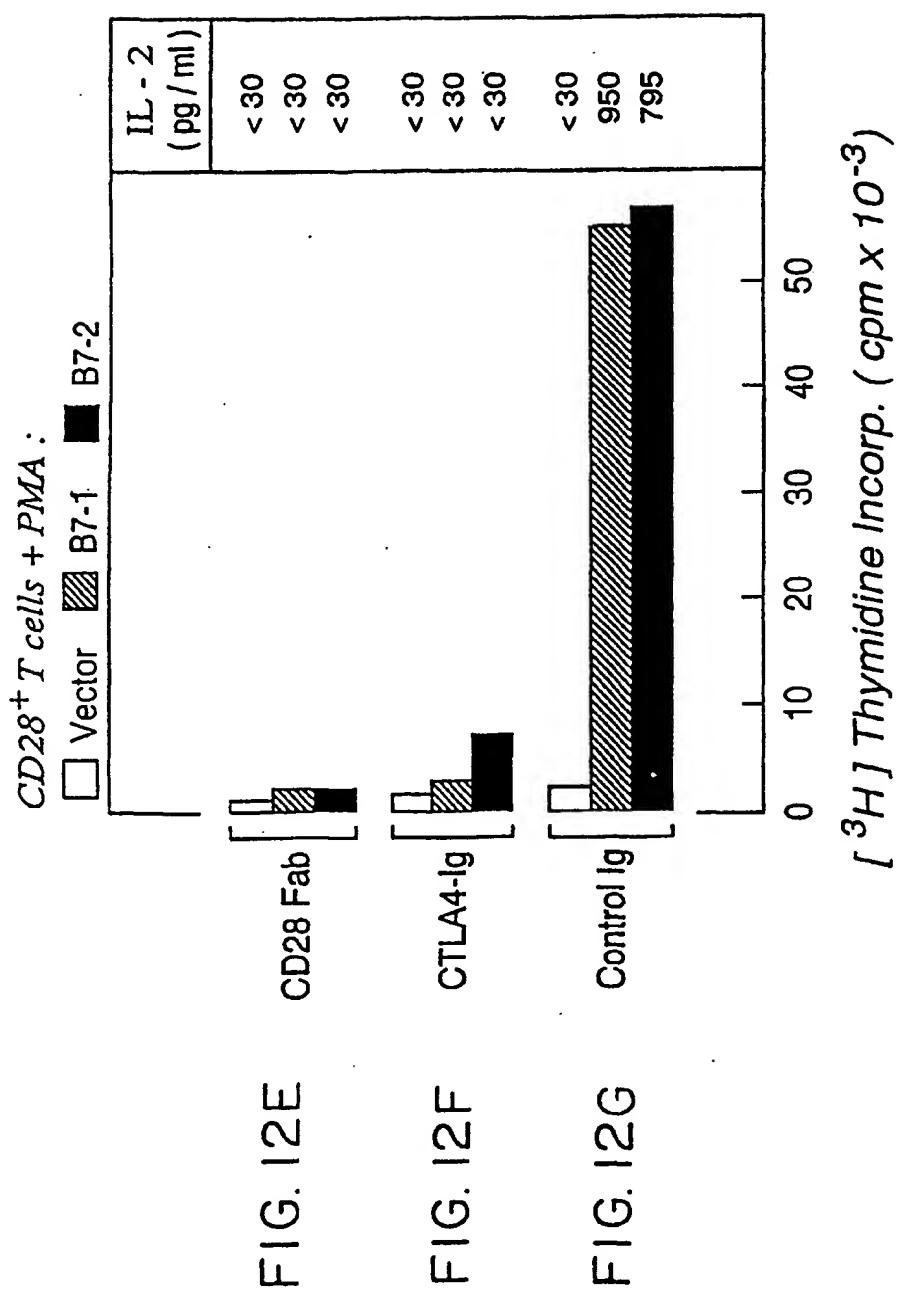
FIG. IIB



14 / 27



15 / 27



16 / 27

FIG. 13A

hB7-1	1	M. . GHTRRQGTPSKCPYLNFFQLLV. LAGLSHFCSGV. IHTKEVREVA	46
hB7-2	1	M. . . . DPQQCTMGLSN. ILFVMAFLLSGA. . . APLKIQAYFNETA	36
mB7	1	MACNCQLMQDTPPLKFPCCPRLLFVILLIRLSQVSSDVDEQLSKSVKDKV	50
hB7-1	47	TLS CGH NVS VEE. LAQTRIY WQKEKKMVL T. MMSGDMNT. . . WPEYKNRT	91
hB7-2	37	DLP CQFANSQNQSLSELVVFWQDQENLVN EYVLGKEKFDSVHSKYMGRT	86
mB7	51	LUF CRY. NSPHEDESEDRIY WQKHDKVVL S. VIAGKLKV. . . WPEYKNRT	95
hB7-1	92	IFDITNL SIVIALRPSDEGT YECVVL KYEKDAFKREHLAEVTLSVKAD	141
hB7-2	87	SFD. SDSWTLRLHNLQIKDKGLYQCIIHHK KPTGMIRIHQMNSELSVLAN	135
mB7	96	LYDNTT. YSLIILGLVLSDRGTYSCVVQKKERGTYEVKHLALVKLSIKAD	144
hB7-1	142	FPTPSISDFEIPTSNI. RRIICSTSGGFPEPH. . . LSWLENGEELNAIN	186
hB7-2	136	FSQPEIVPISNITENVYINTCSSIHGYPEPKMSVLLRTKNSTIEYDGI	185
mB7	145	FSTPNITESGNPSADT. KRITCPASGGFPKP R. . . FSWLENGRELPGIN	189

17 / 27

FIG. 13 B

HB7-1	187	TTVSQDPETELAYAVSSKLDNFN . . MTTNHSFMCLIKYGHLLRVNQTFNWNT	233
HB7-2	186	MQKSQDNNVTELYDVSISSLVSFPDVTSMMTIFCILETDKTRLLSSPFSIE	235
mB7	190	TTISQDPESELYTISSQLDEN . . TTRNHTIKCLIKYGDAHVSEDFTWEK	236
		.	.
		.	.
HB7-1	234	TKQEHF . PDNLPLPSWAITLISVNGIFVICCLTYCFAPRCERRNERLRR	282
HB7-2	236	. LEDPQPPPDPHIPWITAVLP . . . TVIICVMVFCLILWKWKKRPRNSY	280
mB7	237	PPEDPPDSKNTLVLFAGFGAVITVVVIIKCFCKHRSCFRRNAE . SR	285
		.	.
		.	.
HB7-1	283	ESVRPV*	288
HB7-2	281	KCG . . TNTMERESEQTKKREKIHI PERSDEAQRVFKSSKTKSSCDKSDT	327
mB7	286	ETNNNSLTFGPEEAALAEQTVFL *	306
		.	.
HB7-2	328	CG*	329

18 / 27

FIG. 14A

19 / 27

FIG. 14B

TTCTGACGGCACTATTGGGCCACAGAGAAACTTGTGATAGTGTGAATGCCAAGTACCTGG
 301 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 AAGACATGCTCGTATAACCCGGTGTCTCTTTGAACTATCACCTACGGTTCATGGAC
 361 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 L Y E H V L G T E K L D S V N A K Y L G -

 GCCGCACGGCTTGACAGGAACAACGGACTCTACGACTCACAATGTTCAAGATCAAGG
 420 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 CGGGCTGCTCGAAAACGTGTCCTTGTTGAGATGCTGAAGTGTAAAGTCTAGTTTC
 421 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 R T S F D R N N W T L R L H N V Q I K D -

 ACATGGGGCTCGTATGATTGTTATACAAAAAAAGCCACCCACGGATCAATTATCCTCC
 480 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TGTACCCGAGCACTAACAAATATGTTTTCGGTGGGTGTCTAGTTAAATAGGAGG
 481 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 M G S Y D C F I Q K K P P T G S I I L Q -

 AACAGACATTAACAGAAACTGGTGTGCAACTTCAGTGAACCTGAATAAAACTGG
 540 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TTGCTCTGTAATTGGTCTGACAGTCACTAGGGTTGAAGTCACTTGGACTTTTGTGACC
 541 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 Q T L T E L S V I A N F S E P E I K L A -

 CTCAGAAATGTAACAGGAATTCTGGCATAAATTGACCTGCACTGTCACGGTCAAGGTCAAC
 600 +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 GAGTCCTACATTGTCCTTAAGACCGTATTAAACTGGACGTGCAATTGTTCCAGTGG
 601 +-----+-----+-----+-----+-----+-----+-----+-----+-----+

20 / 27

FIG. 14C

CGAAACCTAAGGAGATGTATTTCGTATAACTAATTCAACTAATGGTATGGTATAACA
 601 -----+-----+-----+-----+-----+-----+-----+ 660

GCTTGGATTCTCTACATAAAAGACTATTGATTAAAGTCACTACATACCACTATGT
 661 -----+-----+-----+-----+-----+-----+-----+-----+ 720

R P K K M Y F L I T N S T N E Y G D N M -

TGCGAGATAATGTCACAGAACGTTICAGTATCTCAAACAGCCCTCTCTCTTT
 661 -----+-----+-----+-----+-----+-----+-----+-----+ 720

ACGTCATATAGTGTCTATTACAGTGTCTGACAAGTCATAAGGGTAGGGTGTGGAGAGAAA

Q I S Q D N V T E L F S I S N S L S L S -

CATTCCGGATGGGTGGCATATGACCGTGTGTGTCTGGAAACGGAGTCATGA
 721 -----+-----+-----+-----+-----+-----+-----+-----+ 780

GTAAAGGCCTACCAACACCCGTATACTGGCAACACAGACCTTGCCCTCAGTTACT

F P D G V W H M T V V C V L E T E S M K -

AGATTTCCTCAAACCTCTCAATTCACTCAAGAGTTCCATCTCCTCAAACGTATTGGA
 781 -----+-----+-----+-----+-----+-----+-----+-----+ 840

TCTAAAGGAGGTTGGAGAGTTAAAGTGAGTTCTCAAAGGTAGGGAGTTGCATAACCT

I S S K P L N F T Q E F P S P Q T Y W K -

AGGAGATTACAGCTTCAGTTACTGTGGCCCTCCTTGTGATGCTCATCATTGAT
 841 -----+-----+-----+-----+-----+-----+-----+-----+ 900

TCCTCTTAATGTCGAAGTCATAATGACACCCGGAGGAAACACTACGAGTAGTAACATA

E I T A S V T V A L L V M L L I I V C -

21 / 27

FIG. 14D

GTCACAAAGGCCGAATCAGGCCCTAGCAGGCCAGCAACACAGCCCTCTAAGTTAGAGGGGG
 901 - +-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
 CAGTGTCTTCGGCTTAGTCGGATCGTCCGGGTGTTGGAGATTCAATCTCGCCCC
 H K K P N Q P S R P S N T A S K L E R D -
 ATAGTAACGCTGACAGAGAGACTAACCTGAAGGAACCTGAACCCCAAATTGCTTCAG
 961 - +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 TATCATTTGGCAGCTGTCTCTGTATAAGTGGACTTCCTTGAACTTGGGGTTAACGAAAGTC
 S N A D R E T I N L K E L E P Q I A S A -
 GAAAACCCTATGGCAGGTGAAGGGCAAGTGAGGCCCTGAGGGAAAGAGTTAAAAATTGGCTTG
 1021 - +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 GTTTGGTTTAACGTCTCACTTCCGTCACTCTGGACTCTGGACTCTCAATTAAACGAAAC
 K P N A E *

CCTGAAATAAGAAGTGCAGAGTCTCTCGAAATTCAAATGTTCTCAGCTGATTGGATT
 1081 - +-----+-----+-----+-----+-----+-----+-----+-----+-----+
 GGACTTTATTCTTCAGTCTCAAGAGTCTTAAGTTTACCAAGAGTCGACTAACCTTAAACCTTAA
 1141 - +-----+-----+-----+-----+-----+-----+-----+-----+
 CTACAGTTGAATAATTAAAGAAC 1163
 GATGTCAACTTATTAAATTCTTG

22 / 27

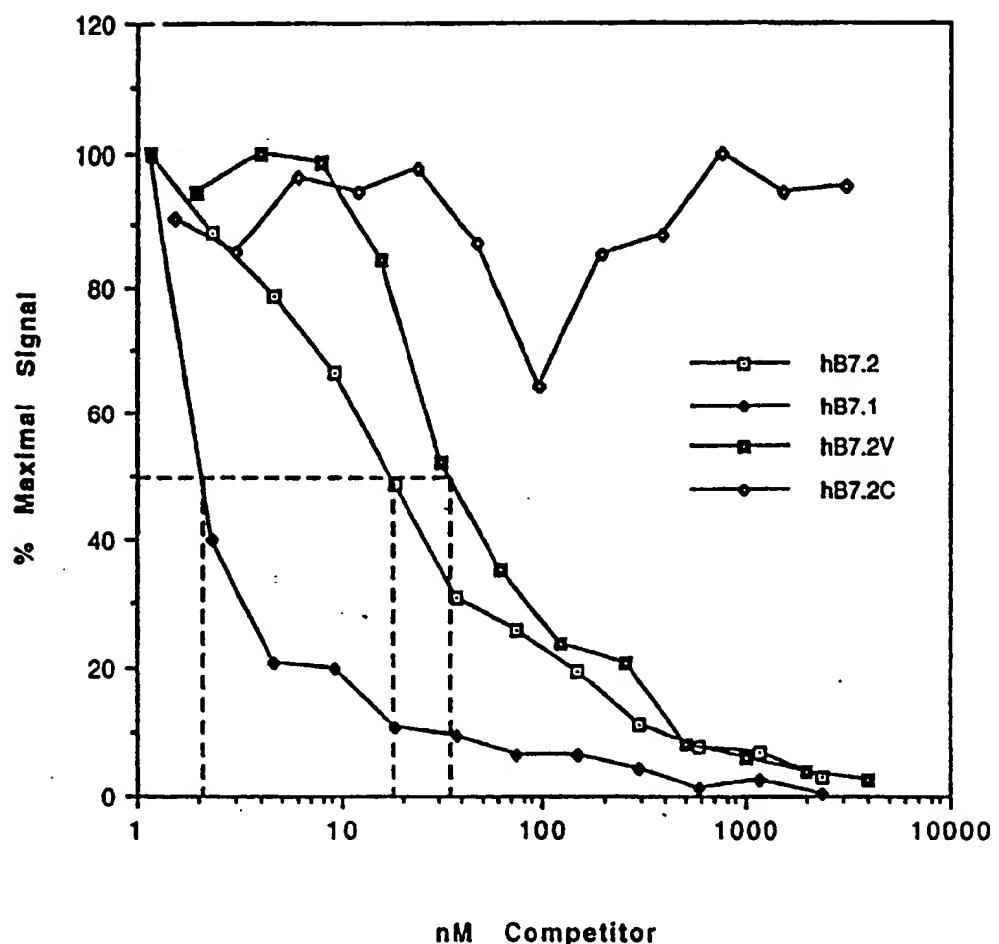


FIG. 15

23 / 27

FIG. 16A

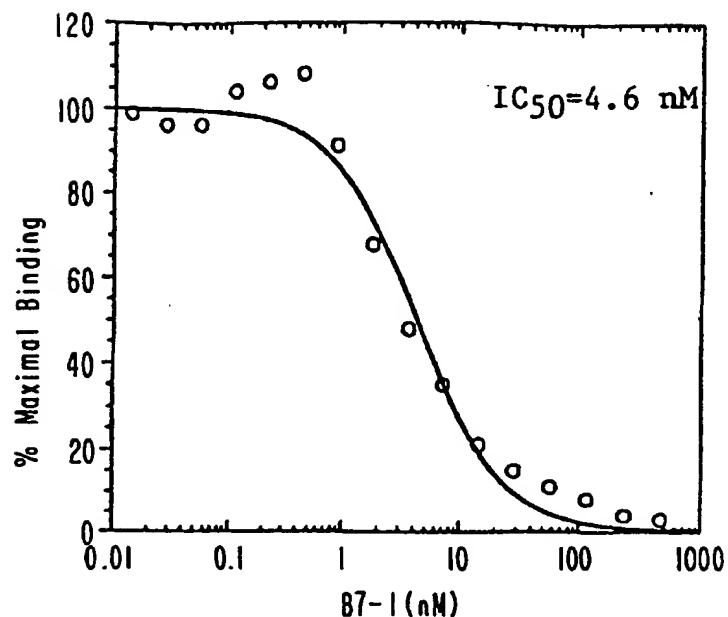


FIG. 16B

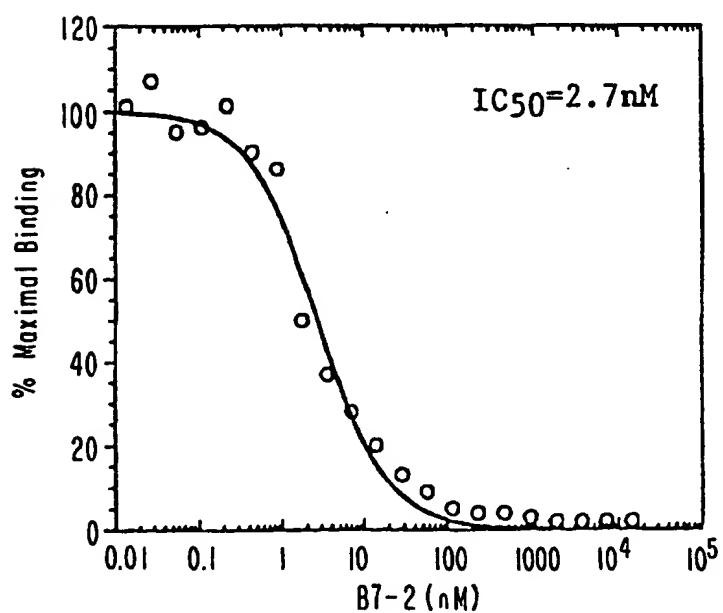


FIG. 17A

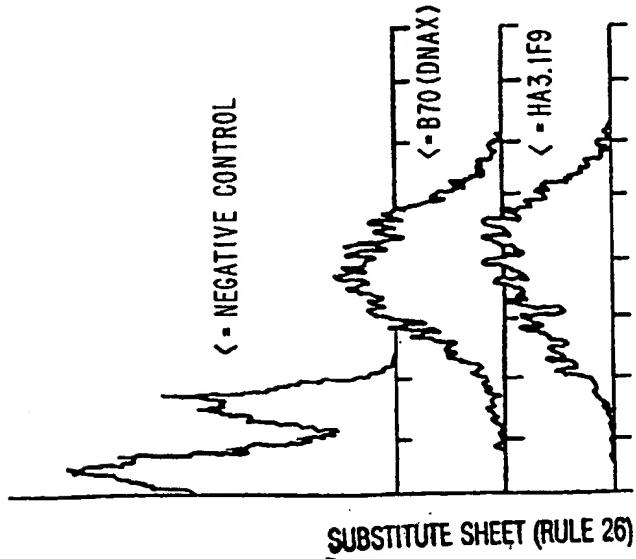


FIG. 17B

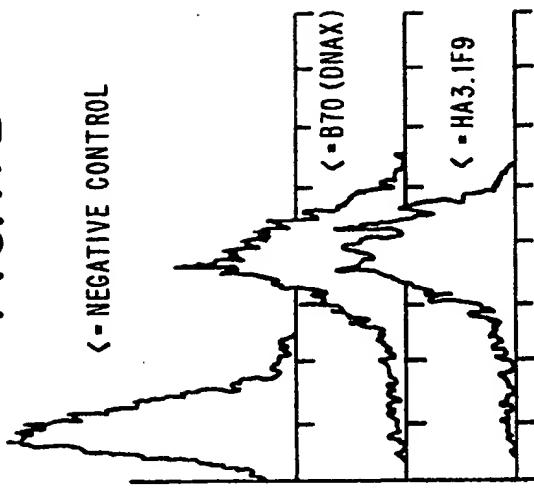
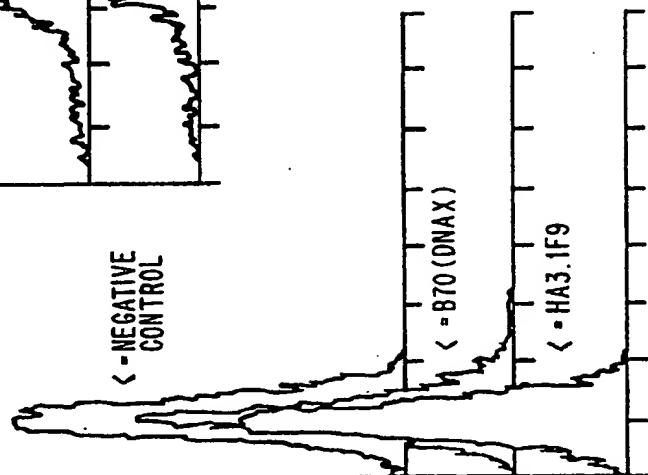
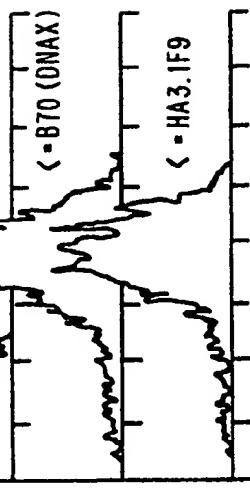


FIG. 17C

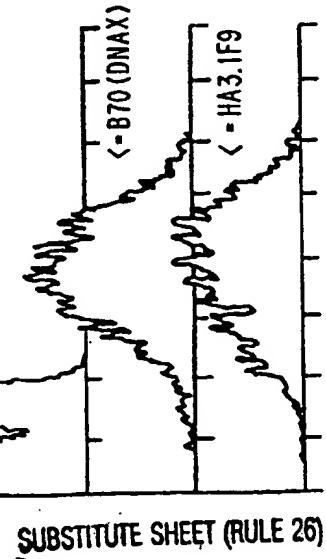


<- NEGATIVE CONTROL

<- NEGATIVE
CONTROL

<- B70 (DNAX)

<- HA3.1F9



SUBSTITUTE SHEET (RULE 26)

25 / 27

FIG. 18B

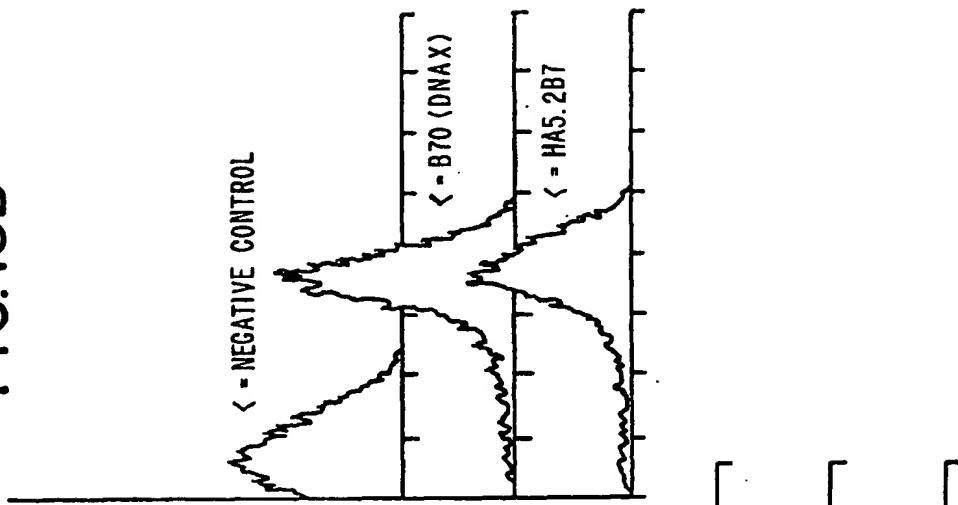
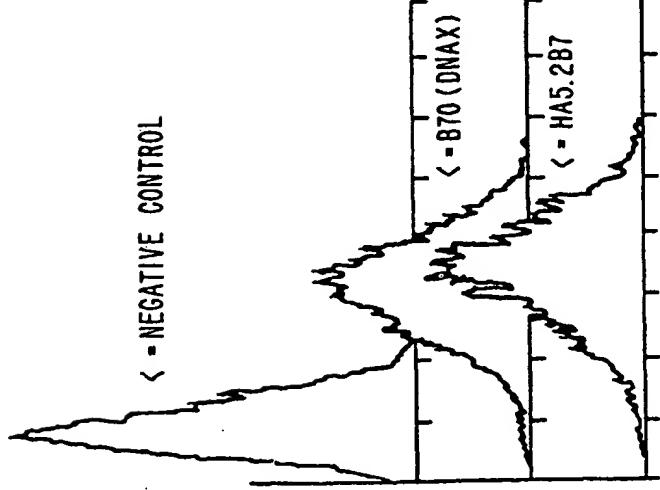


FIG. 18A



SUBSTITUTE SHEET (RULE 26)

FIG. 19A

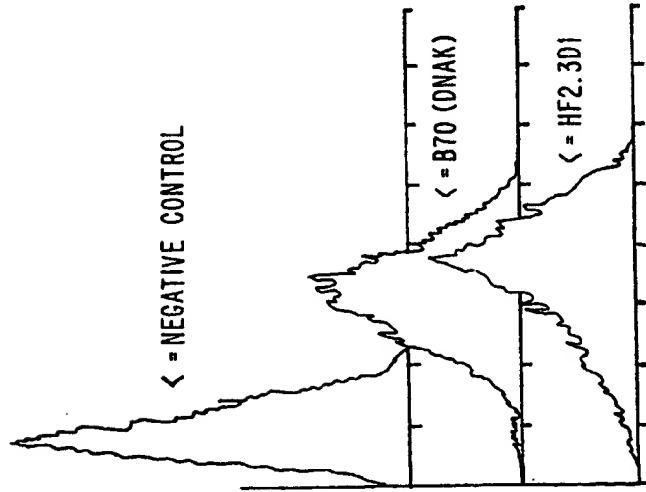
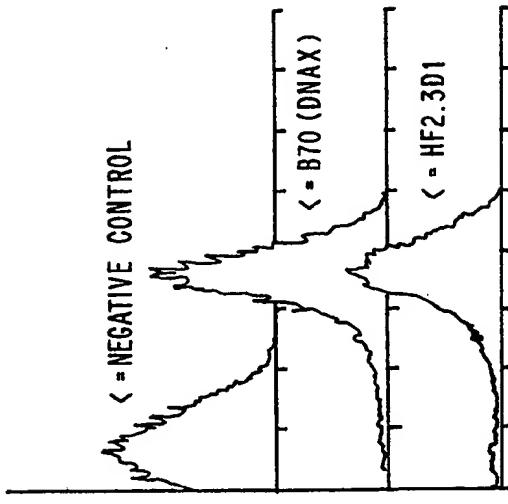
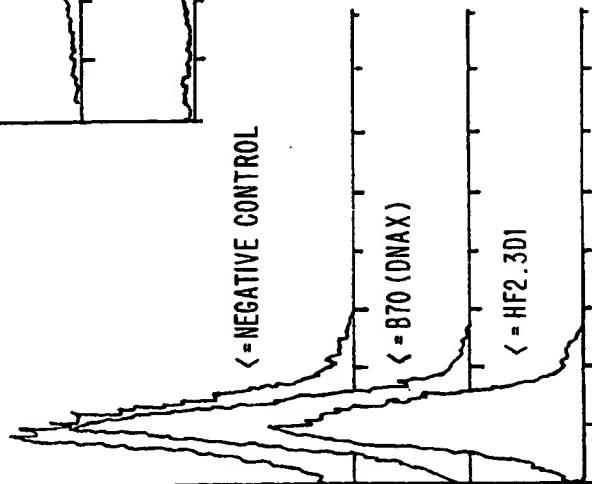


FIG. 19B



< = NEGATIVE CONTROL

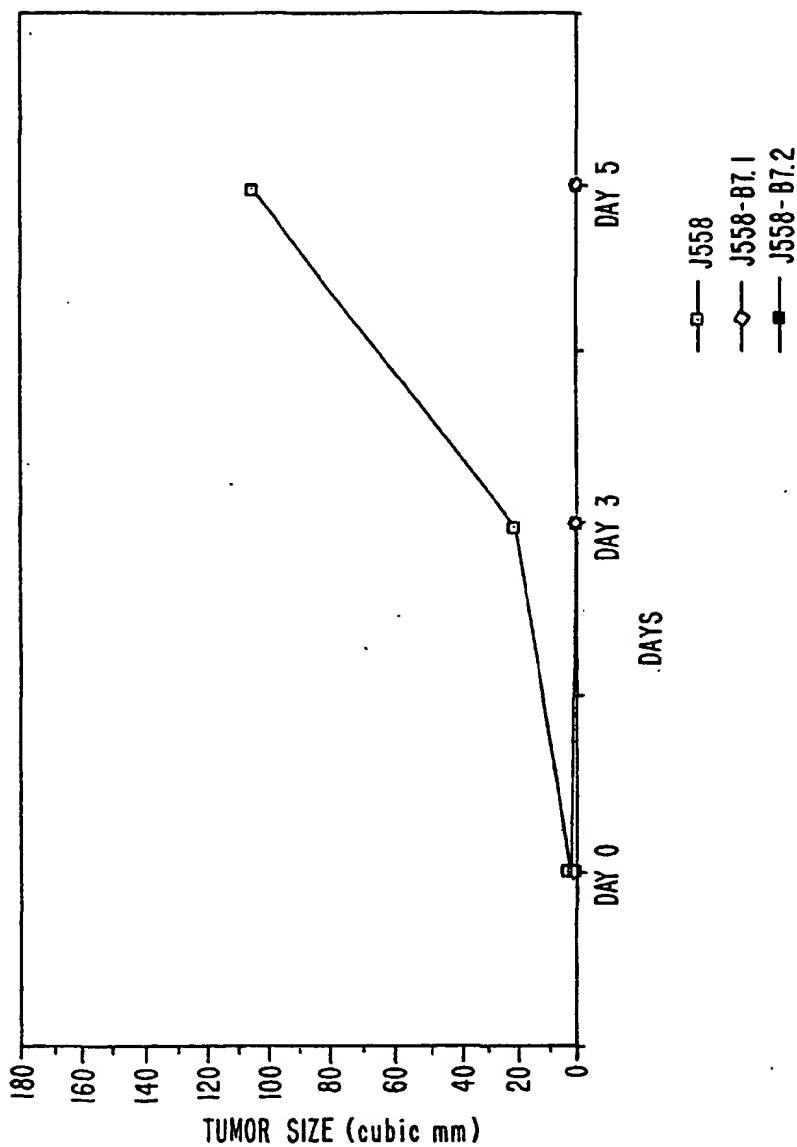
FIG. 19C

< = NEGATIVE CONTROL
< = B70 (DNAX)
< = HF2.3D1

< = NEGATIVE CONTROL

27 / 27

FIG. 20



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/08423

A. CLASSIFICATION OF SUBJECT MATTER				
IPC 6	C12N15/12	C07K14/705	C07K16/28	C12N5/10
	A61K35/12	A61K38/17	A01K67/027	G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOLOGY., vol.149, no.4, 15 August 1992, BALTIMORE US pages 1115 - 1123 AZUMA, M. ET AL.; 'Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by a human natural killer leukemia cell line' ---	
A	WO,A,93 00431 (BRISTOL MYERS SQUIBB) 7 January 1993 ---	
P,X	NATURE., vol.366, 4 November 1993, LONDON GB pages 76 - 79 AZUMA, M. ET AL.; 'B70 antigen is a second ligand for CTLA-4 and CD28' see the whole document ----	1-194

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document number of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

24 November 1994

14-12- 1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl
Fax (+ 31-70) 340-3016

Authorized officer

Nauche, S

INTERNATIONAL SEARCH REPORT

Internat'l Application No
PCT/US 94/08423

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>SCIENCE, vol.262, 5 November 1993, LANCASTER, PA FREEMAN, G.J. ET AL.; 'Cloning of B7-2 : a CTLA-4 counter receptor that costimulates human T cell proliferation.' see the whole document</p> <p>---</p>	1-194
P,X	<p>IMMUNOLOGY TODAY, vol.15, no.7, 1 July 1994, CAMBRIDGE GB pages 321 - 332 JUNE, C.H. ET AL.; 'The B7 and CD28 receptor families.'</p> <p>see the whole document</p> <p>-----</p>	1-43, 48-58, 96-108

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/08423

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **Claims Nos.:**
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 81-95, 138-159, 170-173 are directed to a method of treatment of the human/animal body as well as diagnostic methods and claims 160-169 as far as used in vivo (Rule 39 (iv) PCT) the search has been carried out and based on the alleged effects of the compound/composition.
2. **Claims Nos.:**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. **Claims Nos.:**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Intem: 1 Application No
PCT/US 94/08423

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9300431	07-01-93	AU-A-	2240092	25-01-93
		CA-A-	2110518	07-01-93
		EP-A-	0606217	20-07-94
		JP-T-	6508989	13-10-94
		PT-A-	100637	31-05-94

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT OR DRAWING
- BLURRED OR ILLEGIBLE TEXT OR DRAWING
- SKEWED/SLANTED IMAGES
- COLOR OR BLACK AND WHITE PHOTOGRAPHS
- GRAY SCALE DOCUMENTS
- LINES OR MARKS ON ORIGINAL DOCUMENT
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- OTHER: _____

**IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image
problems checked, please do not report these problems to
the IFW Image Problem Mailbox.**